

# THE BOTANICAL GAZETTE

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EDITOR  
E. J. KRAUS

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VOLUME 102

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WITH THREE PLATES AND FIVE HUNDRED AND THIRTY-TWO FIGURES



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## ERRATA

## VOLUME 101

P. 925, third line from bottom, for "former" read "latter" and on last line, for "latter" read "former"

# THE BOTANICAL GAZETTE

*September 1940*

## DOUBLE FERTILIZATION AND DEVELOPMENT OF THE SEED IN ANGIOSPERMS<sup>\*</sup>

R. A. BRINK AND D. C. COOPER

(WITH NINE FIGURES)

### Introduction

Ever since its discovery by NAVASHIN (19) and GUIGNARD (13) some 40 years ago, double fertilization has remained a puzzling feature of reproduction in the flowering plants. Fertilization of the egg, giving rise to the embryo, is essentially like that in other plants and in animals, both in its morphological and physiological aspects. But conjugation of the second male nucleus with a polar-fusion nucleus to form the primary endosperm nucleus is a phenomenon peculiar to the angiosperms. Three nuclei enter into the structure of the primary endosperm nucleus: a micropylar polar, an antipodal polar, and a sperm nucleus. Participation of a male nucleus in the triple fusion has given rise to much discussion regarding the morphological nature of the resulting endosperm tissue; and the physiological significance of the event has remained almost wholly obscure.

Conjugation of the second male nucleus with the polar-fusion nucleus is interpreted in the present paper as a mechanism by which the developmental advantages of heterosis become available to the endosperm. New evidence supporting the view that the endosperm plays a leading part in the early development of the seed is brought forward; and critical data are presented establishing the fact that, under the particular conditions encountered in flowering plants, the added stimulus to growth associated with hybridity in this tissue may be essential to seed formation.

<sup>\*</sup> Paper from the Department of Genetics, no. 252, Agricultural Experiment Station, University of Wisconsin. The writers appreciate the support received from the Wisconsin Alumni Research Fund and from the WPA Natural Science Project, no. 8649. Examination of relevant histological material from the gymnosperms was made possible through the courtesy of Professor G. S. BRYAN.

Although triploidy, with occasional exceptions, is a salient feature of the endosperm of angiosperms, and the presence of a double complement of maternal chromosomes in the tissue may be important in the intimate reciprocal relationships between endosperm, embryo, and mother plant, these circumstances are outside the boundary of the present discussion. The role of the male nucleus entering into the triple fusion may also be considered apart from them.

COULTER (6), contending that the endosperm is a gametophytic structure, regarded the presence of the male nucleus in triple fusion as of subsidiary importance. This view, however, cannot be maintained in face of the present evidence. Whether the endosperm of angiosperms is more properly regarded as gametophytic than as sporophytic tissue is a morphological problem, and as such does not concern us here. The endosperm in gymnosperms is derived by continuous cell division from the haploid megaspore and is obviously gametophytic. From a functional point of view, interpolation in the angiosperms of a fusion involving a sperm nucleus is a fundamentally significant fact which cannot be ignored in any morphological interpretation. The endosperm is an undifferentiated tissue in gymnosperms and angiosperms alike, and its role is essentially nutritive in both groups. The important thing to recognize is that while a common end is served, the circumstances under which the endosperm functions in the two cases are unlike. The female gametophyte in angiosperms is reduced to its lowest terms, and it is in the light of this fact that the novel mechanism of endosperm formation in flowering plants becomes intelligible.

The distinction which STRASBURGER (22) sought to draw between generative and vegetative fertilization as applying to conjugation of the sperm nuclei with the egg and polar-fusion nuclei, respectively, breaks down in the face of the evidence regarding xenia, as COULTER (6) and EAST (10) have pointed out. Both fertilizations are generative in the sense that the hereditary materials are transmitted in a definite and regular manner. SARGANT (20) has contended that the endosperm remains a formless and frequently short-lived tissue, instead of becoming organized as an embryo, because a vegetative nucleus enters into the fusion which otherwise might be considered sexual. This view, which implies that the endosperm of angiosperms is a degenerate embryo, runs counter to considerable evidence indicating that the endosperm acts as an auxiliary rather than as a competitive factor in embryo development.

The writers (2) have found recently that the low production of seed obtained following self-pollination of *Medicago sativa* is mainly due to two, probably distinct, factors: partial self-incompatibility and high incidence of collapse of fertile ovules in early stages of development. The frequency of fertilization was increased from 14.6 to 66.2 per cent on outcrossing to unrelated plants, and the proportion of fertile ovules collapsing during the first 144 hours after pollination

was reduced from 34.4 to 7.1 per cent. The partial self-incompatibility is not of interest in this connection except as it indicates that alfalfa reproduces largely through cross-pollination. The fact that the rate of collapse of fertile ovules is about five times as high after self-fertilization as after outcrossing is of particular significance. A study of the factors underlying the difference in survival of the two classes of seeds sheds new light on the role of double fertilization in the development of the seed and provides a factual basis for interpreting this hitherto obscure phenomenon.

#### Material and methods

Since the material used and the general methods followed have already been described (5), they will be mentioned only briefly here. Seven alfalfa plants, growing under greenhouse conditions favorable for seed production, were used. The two classes of matings, self-pollination and cross-pollination to unrelated individuals within the group, were made on castrated flowers in different inflorescences under strictly comparable conditions. Pistils were collected at 30, 48, 72, 96, 120, and 144 hours after pollination, and fixed and imbedded in paraffin. After sectioning and staining, the data taken included: fertility of the ovules, frequency of fertile ovules collapsing, number of cells in the proembryo and embryo, and number of nuclei in the endosperm. Alfalfa is well suited to observations of this kind, the ten to twelve ovules per ovary being arranged alternately along the ventral suture, approximately in a straight line. The plane of sectioning can therefore be closely controlled and each ovule critically examined. The number of fertile ovules observed was 433 in the selfed series and 1682 in the crossed series, the two classes being distributed over the seven plants in approximately the same proportion.

When it became evident that the rate of collapse of fertile ovules was markedly different following self- and cross-fertilization, a detailed study of the histological changes associated with failure was undertaken on the material collected from 48 to 144 hours. The changes were found to involve the megagametophyte and the maternal tissue immediately adjacent to it. Each ovule behaved independently at this stage. Except for a slightly greater tendency to persist in the apical region, survival was likewise independent of both position and number of fertile ovules.

It should be emphasized perhaps that the data are based upon more or less direct responses of the ovule to self- and cross-fertilization. The immediate effects of fertilization are local, but they quickly spread to the tissues destined to form the fruit. MURNEEK (18) has shown that as the fruit is stimulated to active growth, profound changes occur also in the general metabolism of the plant. As time elapses beyond fertilization, various secondary changes in the seed may be expected therefore, which add greatly to the difficulties of analysis. The early



post-fertilization stages are of critical importance for survival of the alfalfa seed, and this fact makes this plant favorable material for studying the primary consequences of double fertilization.

### Experimental results

#### COLLAPSE OF FERTILE OVULES

The number of collapsing fertile ovules following self- and cross-fertilization is shown in table 1 and figure 1. Fertilization in alfalfa usually occurs within 30 hours after pollination, and although the frequency is much lower, is only slightly

TABLE 1

FREQUENCY OF FERTILE OVULES COLLAPSING IN SEVEN ALFALFA PLANTS FOLLOWING SELF- AND CROSS-FERTILIZATION. DATA BASED ON COLLECTIONS AT 72, 96, 120 AND 144 HOURS AFTER POLLINATION (AFTER COOPER AND BRINK, 5)

SELF-FERTILIZATION				CROSS-FERTILIZATION			
PLANT SELFED	No. OF FERTILE OVULES		PERCENTAGE COLLAPSING	PLANTS CROSSED	No. OF FERTILE OVULES		PERCENTAGE COLLAPSING
	TOTAL	COLLAPSING			TOTAL	COLLAPSING	
A.....	37	9	24.3	A×B.....	187	13	7.0
B.....	37	19	51.4	B×C.....	110	5	4.5
C.....	20	7	35.0	C×D.....	171	13	7.6
D.....	17	7	41.2	D×E.....	171	16	9.4
E.....	39	8	20.5	E×A.....	146	9	6.2
F.....	109	39	35.8	F×G.....	228	14	6.1
G.....	55	19	34.5	G×F.....	198	16	8.1
Total....	314	108	34.4	Total....	1211	86	7.1

delayed after selfing as compared with crossing. Observations at 48 hours after pollination afford little evidence of ovule degeneration, but at 72 hours the collapse of fertile ovules is common. From previous data (5) on seed production on these plants, it may be estimated that about 20 per cent of the reduction in functioning ovules following cross-fertilization and about 70 per cent following self-fertilization occurring between fertilization and maturity of the seed takes place in the approximately 100-hour period on which table 1 is based. Collapse is not therefore limited to the relatively brief interval during which the samples were taken; but it is evident that the period is critical for seed survival, particularly after self-pollination.

In each of the seven plants the percentage of fertile ovules collapsing is much higher following self-pollination than after crossing, the respective mean values for the two groups being 34.4 and 7.1. Since the matings were made concurrently

on the same individuals, the disparity in survival evidently is attributable to the difference in genetic composition of the embryos and endosperms within the two classes of seeds.

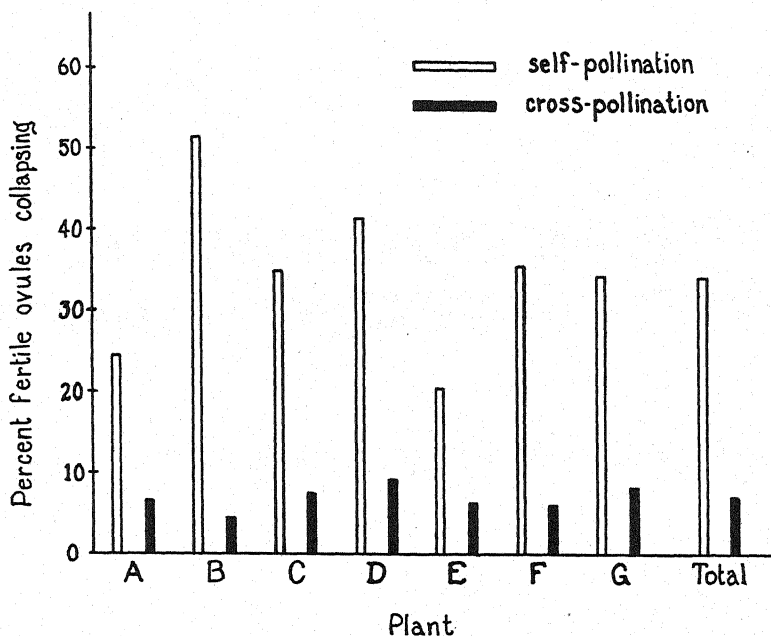


FIG. 1.—Frequency of fertile alfalfa ovules collapsing in pistils collected at 72, 96, 120, and 144 hours following self- and cross-pollination (after COOPER and BRINK, 5).

#### HISTOLOGY OF FERTILE OVULE COLLAPSE

Just prior to and at the time of fertilization in alfalfa there is little, if any, meristematic activity in any portion of the ovule, the nuclei being in a resting state.

When fertilization does not occur there is no further development of the ovule. The latter retains its shape and appears potentially functional for another 24 hours, then degenerates. The first evidence of breakdown is collapse of the megagametophyte in the region of the bend of the ovule. The collapse is accompanied by shrinkage of the ovule in that region. Disintegration of the megagametophyte and ovule continues, so that the infertile structure is much shrunken at 96 and 120 hours after pollination. Following fertilization, on the other hand, the entire ovule is immediately stimulated to develop. Active nuclear and cell division is initiated in the integuments and funiculus. The few remaining cells of the nucellus at the chalazal end of the megagametophyte do not divide further. They persist for a time, evidence of them being found in some seeds up to 144 hours after pollination. Ultimately these cells disintegrate completely and the endosperm comes to lie in direct contact with the inner integument.

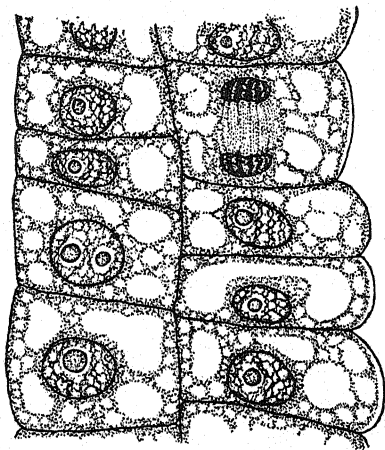
The primary endosperm nucleus divides shortly after fertilization, the position of the spindle being oriented so that each arm of the elongated V-shaped mother cell receives one of the daughter nuclei. Further divisions occur, the endosperm becoming multinucleate. Later, 6-7 days after pollination, cell formation is initiated in the region of the embryo and advances toward the chalaza, so that in a short time the endosperm is completely cellular. As long as the endosperm is in a free nucleate condition the number of nuclei in each arm is approximately equal. At these early stages of development the nuclei are located in the peripheral layer of cytoplasm which surrounds the elongated central vacuole.

Nuclear and cell divisions continue in the outer integument and funiculus, so that these structures become both longer and larger as development proceeds. The cells of the inner integument become highly vacuolate shortly after fertilization has taken place and have the appearance of tapetal cells. They likewise continue to divide, but the plane of division is such that the integument increases in length but remains two layers of cells in thickness. Occasionally a cell may divide in the opposite plane, and at that point the integument will be three cells thick.

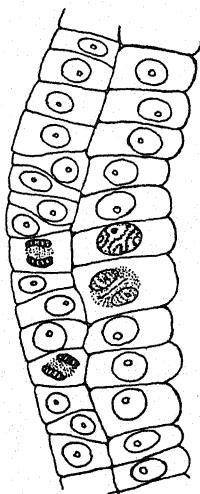
The cells of the proembryo at the various stages of development are densely cytoplasmic and stain heavily, especially those near the apex. After formation of the true embryo, the basal cells of the suspensor become more vacuolate and stain lightly, whereas those of the embryo remain dense and stain heavily.

The first evidence of collapse of a fertile ovule may be seen approximately 48 hours after pollination. The cells of the inner integument in the chalazal arm on the side adjacent to the funiculus become more or less densely cytoplasmic and assume a meristematic appearance (fig. 5), instead of remaining tapetum-like as in the normally developing seed (fig. 2). Active nuclear and cell division is initiated in this region (fig. 6). The mitotic figures are oriented at various angles and cell plate formations are in diverse planes, so that a number of layers of cells result (fig. 7). In a normally developing seed the plane of cell division in this region of the inner integument is usually longitudinal (fig. 3), and for the most part two layers of cells are formed (fig. 4).

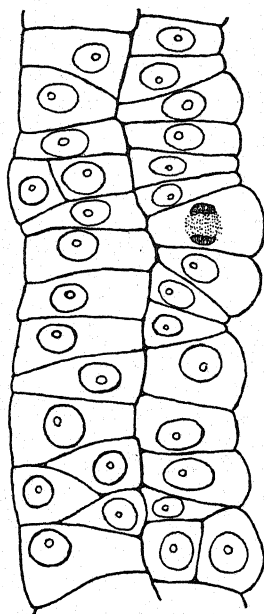
During the course of this localized meristematic activity of the inner integument, the few remaining nucellar cells increase in size and stain heavily. The endosperm collapses in a region just posterior to the bend of the seed and shrinks away from the actively proliferating portion of the integument. The collapse proceeds toward both ends of the seed. The chalazal end of the endosperm collapses first and may be completely disorganized while the micropylar end may still be more or less turgid. The cells of the embryo in such a seed become highly vacuolate and starved in appearance. Shortly thereafter the remaining endosperm and the embryo become disorganized. Although the fertile ovule ceases further development, it retains its shape more or less for some time before collapsing. Such collapsing seeds are common after selfing and of infrequent occurrence following crossing.



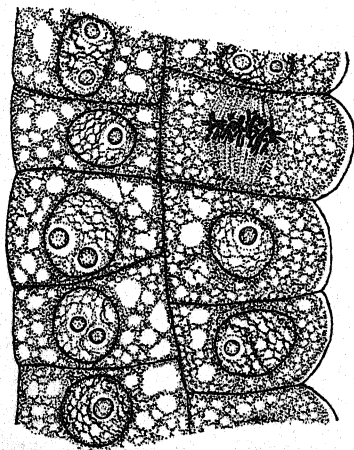
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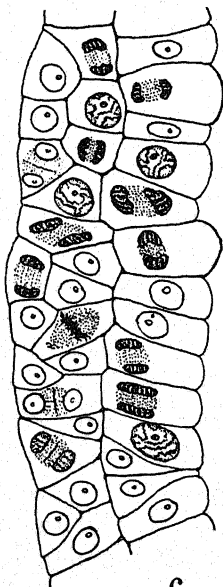
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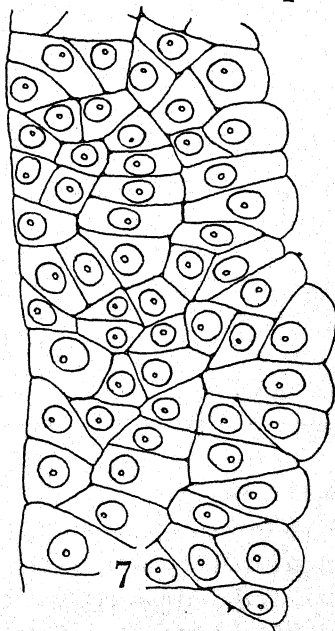
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5



6



7

FIGS. 2-7.—Fig. 2, portion of inner integument from chalazal region of fertile ovule adjacent to funiculus, showing highly vacuolate condition of cells 48 hours following cross-pollination. Fig. 3, same 72 hours after cross-pollination. Fig. 4, 96 hours after cross-pollination; inner integument remains two layers of cells in thickness. Fig. 5, portion of inner integument from chalazal region of fertile ovule adjacent to funiculus, showing dense cytoplasm of cells 48 hours after self-pollination. Fig. 6, same 72 hours after self-pollination, showing large number of cells in stages of division. Fig. 7, 96 hours after self-pollination; integument now composed of several layers of cells.

## RESERVE FOODS IN OVULE

The mature ovule of alfalfa is almost completely lacking in reserve foods. No storage products are to be seen in the main body of the ovule, and the small amount of starch within the megagametophyte quickly disappears after fertilization. Henceforth the young seed is dependent upon nutrients moved in from adjacent parts of the plant.

Development of the ovule and young seed at a nutrient level so low that little storage occurs appears to be significant in relation to survival. Failure of the fertile ovule to continue development frequently may be due to starvation as a result either of shortage of supply or unbalanced distribution of the nutrients between the tissues inside and outside the endosperm.

Examination was made of the mature ovules of thirty-six additional species of angiosperms, representing fifteen families, to determine whether the paucity of stored foods in the ovule was characteristic of the flowering plants. The observations, summarized in table 2, show that the condition found in alfalfa is more or less typical of the whole group.

Some starch was found in the ovules of twenty-one of the thirty-six species. It is usually limited to the egg and the endosperm mother cell of the megagametophyte. The amount in these cells varies from very little in *Ulmus*, *Lupulinus*, *Lycopersicum*, and some others to an abundance in *Zea*, *Melilotus*, *Phryma*, and certain additional species. Reserve foods were not found in the synergids; and in only one species, *Amphicarpa*, was starch detected in the antipodals.

Storage was observed in other parts of the ovule in only three species. The nucellus and integuments contained starch in *Trigonella* sp., abundant food was present in the nucellus in *Galega officinalis*, and in *Phryma leptostachya* a limited amount of storage material was found in the outer integument.

Post-fertilization stages of seventeen of the twenty-one species wherein some food materials are stored in the ovule showed that such materials are usually consumed by the time a 2-celled proembryo is present; in only one species, *Galega officinalis*, was there evidence of stored foods beyond the 4-celled proembryo stage. Some starch was present in the nucellus of ovules of *Galega* containing embryos consisting of four cells plus suspensor. The starch in the megagametophyte was completely consumed at the 2-celled proembryo stage.

Generally speaking, therefore, the mature ovule of angiosperms is characterized by little or no stored foods. The limited nutrient reserves occurring in most species usually do not suffice beyond early post-fertilization development. DAHLGREN (9) found that storage of starch in the megagametophyte seems to attain its maximum immediately before fertilization. The grains disappear rapidly thereafter.

TABLE 2  
RELATIVE ABUNDANCE AND DISTRIBUTION OF STORED FOODS IN MATURE  
OVULES OF 37 SPECIES OF ANGIOSPERMS

SPECIES	MEGAGAMETOPHYTE				OVULE		
	EGG	ENDO- SPERM MOTHER CELL	SYNER- GIDS	ANTI- PODALS	NUCEL- LUS	INNER INTEGU- MENT	OUTER INTEGU- MENT
Gramineae							
Euchlaena mexicana.....	+++*	+++	-	-	-	-	-
Zea mays.....	+++	+++	-	-	-	-	-
E. mexicana × Z. mays.....	+++	+++	-	-	-	-	-
Commelinaceae							
Commelina communis.....	-	-	-	-	-	-	-
Liliaceae							
Erythronium americanum.....	-	-	-	-	-	-	-
Lilium martagon.....	-	-	-	-	-	-	-
L. regalis.....	-	-	-	-	-	-	-
L. henryi.....	-	-	-	-	-	-	-
Urticaceae							
Ulmus americana.....	+	+	-	-	-	-	-
Amarantaceae							
Amaranthus graecizans.....	-	-	-	-	-	-	-
A. retroflexus.....	-	-	-	-	-	-	-
Portulacaceae							
Portulaca oleracea.....	-	-	-	-	-	-	-
Cruciferae							
Brassica oleracea.....	++	++	-	-	-	-	-
Leguminosae							
Melilotus albus.....	+++	+++	-	-	-	-	-
Medicago sativa.....	++	++	-	-	-	-	-
Medicago sativa (4n).....	+++	+++	-	-	-	-	-
Medicago lupulina.....	++	++	-	-	-	-	-
Medicago glutinosa.....	++	++	-	-	-	-	-
Lupulinus polyphyllous.....	+	+	-	-	-	-	-
Desmodium grandiflorum.....	-	-	-	-	-	-	-
Lathyrus latifolius.....	-	-	-	-	-	-	-
Amphicarpa monoica.....	++	++	-	++	-	-	-
Galega officinalis.....	+++	+++	-	-	+++	-	-
Trigonella sp.....	Oil	Oil	-	-	++	++	++
Pisum sativum.....	-	-	-	-	-	-	-
Phaseolus vulgaris.....	-	-	-	-	-	-	-
Malvaceae							
Abutilon theophrasti.....	-	-	-	-	-	-	-
Solanaceae							
Nicotiana rustica.....	++	++	-	-	-	-	-
Lycopersicum esculentum.....	+	+	-	-	-	-	-

\* +++ = Abundant.  
 ++ = Medium.  
 + = Little.  
 - = None.

TABLE 2—*Continued*

SPECIES	MEGAGAMETOPHYTE				OVULE		
	EGG	ENDO-SPERM MOTHER CELL	SYNERGIDS	ANTI-PODALS	NUCEL-LUS	INNER INTEGUMENT	OUTER INTEGUMENT
Scrophulariaceae							
Chelone glabra.....	+	+	—	—	—	—	—
Phrymaceae							
Phryma leptostachya.....	+++	+++	—	—	—	+	—
Dipsacaceae							
Scabiosa japonica.....	++	++	—	—	—	—	—
Scabiosa atropurpurea.....	++	++	—	—	—	—	—
Cucurbitaceae							
Cucumis sativa.....	+	+	—	—	—	—	—
Compositae							
Solidago canadensis.....	—	—	—	—	—	—	—
Cichorium intybus.....	—	—	—	—	—	—	—
Sonchus oleraceus.....	—	—	—	—	—	—	—
Taraxacum officinalis.....	—	—	—	—	—	—	—

## GROWTH OF EMBRYO

At 30 hours after pollination a few zygotes have divided, and at 48 hours this is the modal condition. The endosperm often contains eight or sixteen nuclei at this time. Transverse division of the apical cell of the proembryo continues until a row of six cells is formed. The apical cell then divides longitudinally, giving rise to the first two cells of the embryo. The number of cells can be accurately counted up to 144 hours after pollination. A few divisions may occur in the suspensor during early differentiation of the embryo, leading sometimes to ten cells in this structure.

The counts on number of cells in the proembryo and embryo plus suspensor at six periods following self- and cross-fertilization are summarized in table 3. The data, like those on the endosperm to follow, were taken only on seeds showing no signs of disintegration. The mean values are the basis of the two growth curves in the lower part of figure 8.

Two facts are apparent from the data in table 3. In the first place, the number of cells in the embryo during the interval covered increases at approximately the same rate following both self- and cross-fertilization. The hybrid embryos may have a slight advantage, but the difference is not significant. Secondly, the growth curves depart only slightly from linearity; in other words, the number of cells increases additively.

TABLE 3  
NUMBER OF CELLS IN THE EMBRYO FOLLOWING SELF- AND CROSS-FERTILIZATION

TIME IN HOURS AFTER POLLINATION	TYPE OF MATING	NUMBER OF CELLS IN EMBRYO																																								TOTAL	AVERAGE			
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40			41	42	
30.....	{Self {Cross	78 252	3 7	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	81 259	1.04 1.03	
48.....	{Self {Cross	33 70	79 220	4 16	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	116 312	1.75 1.81	
72.....	{Self {Cross	.....	16 21	20 52	26 145	8 39	4 24	3 15	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	77 302	3.65 4.16	
96.....	{Self {Cross	.....	1 4	2 12	8 51	5 190	7 1	44 39	3 1	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	75 310	6.63 7.08	
120.....	{Self {Cross	.....	.....	.....	.....	.....	17 73	2 5	16 16	2 4	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	39 234	9.31 10.32
144.....	{Self {Cross	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	45 265	13.80 17.28



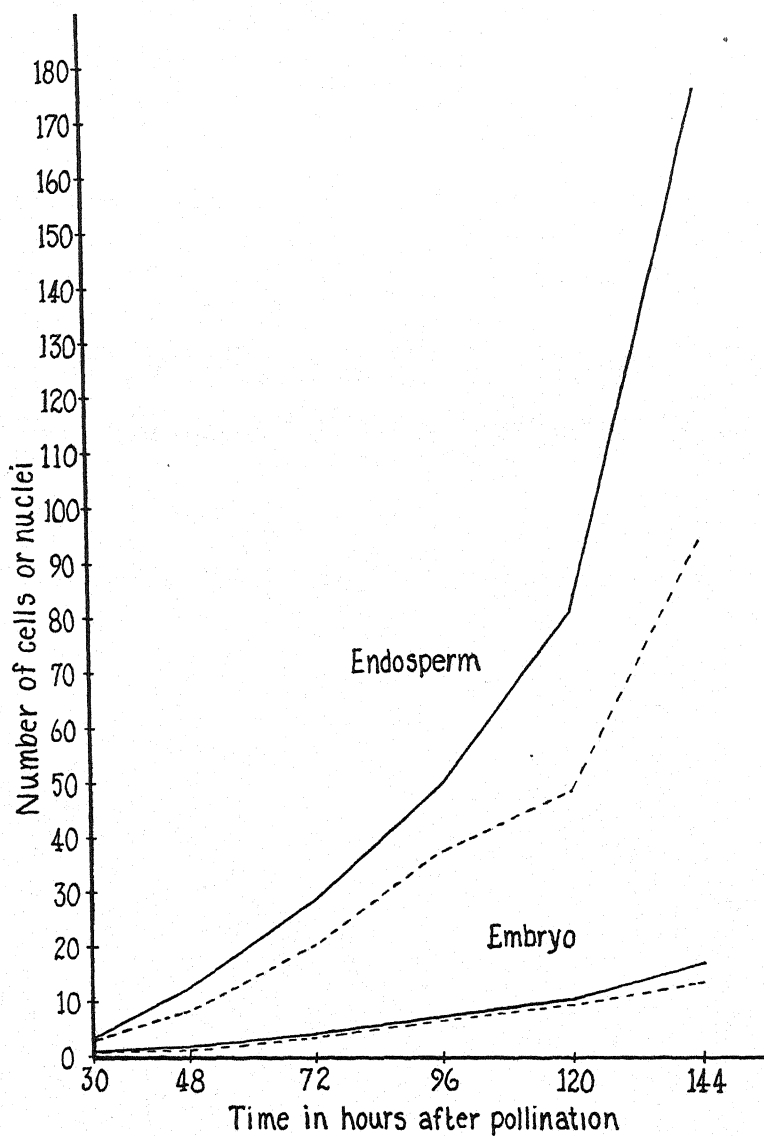


FIG. 8.—Increase in number of cells in embryo and in number of nuclei in endosperm following self- (broken line) and cross-fertilization (continuous line).

## GROWTH OF ENDOSPERM

The endosperm of alfalfa grows by free nuclear division during the period under observation. Only at 144 hours after pollination is the beginning of wall formation to be observed in a few seeds. Division is synchronous in the nuclei lying scattered throughout the cytoplasm. Starting at the chalazal end, a transient wave of mitosis progresses toward the micropylar end. Nuclear increase thus proceeds in a series of pulsations, as it were, each surge doubling the number. The significance of the rhythmic behavior is not known, but it demonstrates that the whole endosperm functions as a physiological unit at this stage, as might not be the case if the tissue were cellular. It may be noted also that division in unison is a mechanism admirably adapted to support inherent and dependent metabolic processes at a maximum potential. This characteristic of the endosperm may be important in relation to growth in the surrounding maternal tissue, as discussed later.

Growth in the endosperm was recorded in terms of the number of nuclei present. It might be objected that the data are not comparable with those from the embryo where cell formation occurs from the beginning. In a measure this criticism is well founded, and there is the further reservation that in neither case is the actual change in mass of tissue measured. Justification for considering that the bases used in comparing rates of development of embryo and endosperm are reasonably alike is seen in the fact that the latter shortly becomes cellular, the number of cells formed being equal to the number of nuclei present.

The data on growth of the endosperm following self- and cross-fertilization are shown in table 4. The mean numbers of nuclei present in the two series at 30, 48, 72, 96, 120, and 144 hours after pollination are plotted to form the two upper curves in figure 8. Except at 144 hours, the values were obtained by counting the nuclei present in each endosperm. At 144 hours the number is sometimes so large that it is not only difficult but also extremely laborious to determine. The difficulty is increased if cell-wall formation has started. The actual number of nuclei present was determined in the 144-hour material, however, in all cases where development of the embryo had not advanced beyond the 4-celled stage. This group comprised about one-third the seeds in the selfed series and about one-fifth those in the crossbred group. The number of nuclei in the remaining 144-hour seeds was estimated by averaging the counts from a part of the endosperms in each series distributed in accordance with the frequencies of the different embryo size classes. It is thought that the mean values thus obtained represent with sufficient accuracy for present purposes the stage of endosperm development in the more advanced seeds.

The fact has long been known that, following fertilization in angiosperms, the endosperm develops much more rapidly than the embryo. Few satisfactory meas-

urements of rate of endosperm development have been made, and we know of no other body of evidence as extensive as the present one. The data clearly confirm the established view that in rate of activity the endosperm far exceeds the embryo at this stage. A further conclusion may now be drawn. Whereas the number of cells in the young embryo increases approximately in arithmetical progression, the number of nuclei in the endosperm tends to increase geometrically. How long this relation is maintained in the growth of the alfalfa seed and whether it occurs in plants where the endosperm is cellular from the start is not known. As a

TABLE 4  
NO. OF NUCLEI IN ENDOSPERM FOLLOWING SELF- AND CROSS-FERTILIZATION

TIME IN HOURS AFTER POLLINA- TION	TYPE OF MATING	NO. OF NUCLEI IN ENDOSPERM														TOTAL	AVERAGE
		1	2	4	8	16	32	64	96	128	192	256	384	512			
30.....	{Self	11	30	37	3	.....	.....	.....	.....	.....	.....	.....	.....	.....	81	3.0	
	{Cross	25	46	151	37	.....	.....	.....	.....	.....	.....	.....	.....	.....	259	3.9	
48.....	{Self	8	4	16	67	21	.....	.....	.....	.....	.....	.....	.....	.....	116	8.2	
	{Cross	5	6	36	76	183	6	.....	.....	.....	.....	.....	.....	.....	312	12.5	
72.....	{Self	.....	.....	5	8	37	26	1	.....	.....	.....	.....	.....	.....	77	20.4	
	{Cross	1	2	2	13	80	183	20	1	.....	.....	.....	.....	.....	302	28.6	
96.....	{Self	.....	.....	.....	1	15	38	21	.....	.....	.....	.....	.....	.....	75	37.4	
	{Cross	.....	.....	.....	2	8	134	160	1	5	.....	.....	.....	.....	310	49.7	
120.....	{Self	.....	.....	.....	.....	4	18	13	3	1	.....	.....	.....	.....	39	48.4	
	{Cross	.....	.....	.....	.....	.....	27	112	48	44	3	.....	.....	.....	234	80.5	
144.....	{Self	.....	.....	.....	.....	.....	4	14	11	12	2	.....	.....	.....	43	93.0	
	{Cross	.....	.....	.....	.....	.....	.....	4	7	38	16	16	4	1	86	174.5	

description of the present observations, however, it fits reasonably well. The implication of the fact is that of the two products of the fertilization process, embryo and endosperm, it is the endosperm which is the ascendant tissue in early development of the seed.

The relative development of the endosperm following self- and cross-fertilization may next be compared. As shown in figure 8, more nuclei occur in the hybrid endosperms (solid line) than in the inbred endosperms (broken line) at each of the intervals studied. Since the pistils collected each time necessarily were different, the data may be considered as comprising six independent sets of paired observations. If random sampling alone were operative in determining the sign of the difference in mean number of nuclei in the two series, one would expect the sign to be the same in all six cases only once in sixty-four trials. It is probable,

therefore, that the rate of growth of the endosperm following hybridization is significantly higher than after self-fertilization.

Increase in rate of growth of the young endosperm following entrance into the triple fusion of a male nucleus from another plant is a fact of importance in understanding double fertilization. Its full significance becomes clear when considered in relation to the histological changes associated with seed collapse just presented.

NUMBER OF ENDOSPERM NUCLEI ASSOCIATED WITH EMBRYOS AT  
DIFFERENT STAGES OF DEVELOPMENT

Fertilization after self-pollination in alfalfa may be somewhat deferred, owing to partial self-incompatibility. The delay is known to be rather brief. Neverthe-

TABLE 5

NO. OF ENDOSPERM NUCLEI ASSOCIATED WITH EMBRYOS OF GIVEN SIZES AFTER  
SELF- AND CROSS-FERTILIZATION. DATA BASED ON 30-144 HOUR MATERIAL

STAGE OF EMBRYO DEVELOPMENT	TYPE OF MATING	NO. OF NUCLEI IN ENDOSPERM										TOTAL	AVERAGE
		1	2	4	8	16	32	64	96	128	192		
1-celled proembryo . . . . .	{Self	18	34	47	12	...	...	...	...	...	...	111	3.3
	{Cross	30	52	183	64	2	1	...	...	...	...	332	4.3
2-celled proembryo . . . . .	{Self	...	...	10	63	26	...	...	...	...	...	99	9.7
	{Cross	1	2	6	55	180	4	...	...	...	...	248	14.0
3-celled proembryo . . . . .	{Self	1	...	1	2	16	6	...	...	...	...	26	18.0
	{Cross	...	...	...	8	43	21	...	...	...	...	72	19.8
4-celled proembryo . . . . .	{Self	...	...	...	1	18	15	...	...	...	...	34	22.8
	{Cross	...	...	...	1	40	116	...	...	...	...	157	27.8
5-celled proembryo . . . . .	{Self	...	...	...	1	3	8	1	...	...	...	13	28.9
	{Cross	...	...	...	...	2	41	8	...	...	...	51	36.4
6-celled proembryo . . . . .	{Self	...	...	...	...	5	5	1	...	...	...	11	27.6
	{Cross	...	...	...	...	2	51	22	...	...	...	75	41.0
2-celled embryo+suspen- sor . . . . .	{Self	...	...	...	...	9	38	20	...	...	...	67	39.4
	{Cross	...	...	...	...	2	106	159	14	3	...	284	54.0
3-celled embryo+suspen- sor . . . . .	{Self	...	...	...	...	...	2	9	...	...	...	11	58.2
	{Cross	...	...	...	...	...	...	5	1	...	...	6	69.3
4-celled embryo+suspen- sor . . . . .	{Self	...	...	...	...	...	10	10	2	4	...	26	64.0
	{Cross	...	...	...	...	...	11	114	28	46	4	203	83.7

less it may be long enough to raise doubt concerning the validity of comparing embryo and endosperm development after self- and cross-fertilization on a basis in which time of pollination is used as the zero point. Comparison of the growth relations independently of time as a coordinate is therefore desirable.

The number of nuclei in the endosperm in relation to stage of development of the associated embryo is shown in table 5 and figure 9. The primary data are the same as those in tables 2 and 3, except that seeds in which the true embryo has advanced beyond the 4-celled stage are not included. As mentioned earlier, the number of nuclei in endosperms associated with embryos comprising five cells or more were estimated by averaging the values from a part of the seeds.

It will be seen from table 5 and figure 9 that at all nine stages of embryo development—zygote to 4-celled true embryo—the number of nuclei in the endosperm is

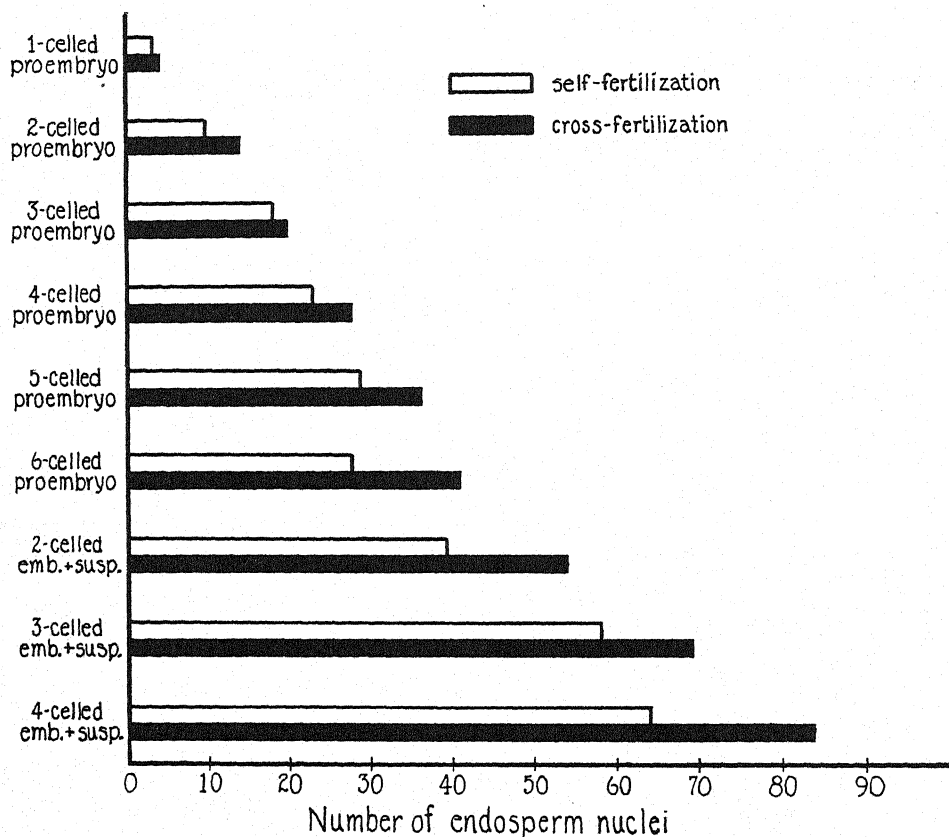


FIG. 9.—Number of endosperm nuclei associated with proembryos and embryos at various stages of development following self- and cross-fertilization.

larger after cross- than after self-fertilization. This evidence therefore confirms the conclusion already stated, that following hybridization the endosperm grows more rapidly than after selfing. Likewise it directly establishes the important corollary that, at a given early stage of development, a hybrid embryo is accompanied by a more advanced endosperm than is an inbred one.

### Discussion

Double fertilization is of almost universal occurrence in angiosperms. According to SCHNARF (21), even in the few groups—such as the Helobiae and orchids—in which the endosperm may be regarded as suppressed, conjugation of the second male nucleus with the polar-fusion nucleus probably does not always fail. Permanency of the fully developed endosperm, however, varies greatly between species. The endosperm may be consumed by the embryo during development of the seed, as in most Leguminosae, including *Medicago sativa*; or it may persist as a constituent of the mature seed, as in the Gramineae, serving to nourish the seedling. There is general agreement that the endosperm is essentially a nutritive tissue, but its relation to the embryo in this respect during early post-fertilization development seems to have received little attention. The place of the fully developed endosperm in the economy of the seed is obvious, but the scanty attention given the tissue in embryological studies has allowed the more general and fundamental role in the young seed to remain in obscurity.

Four lines of evidence indicate that the endosperm of angiosperms exercises a primary and essential function in the early development of the seed. These may be summarized as follows:

1. Whatever its duration may be, endosperm tissue is regularly present at this stage. It is significant that in the rare exceptions to this rule, special devices may occur tending to serve the same end. In the Orchidaceae, for example, the suspensor of the embryo may serve as a haustorium, in certain cases growing out of the micropyle, branching, and imbedding itself in adjacent nutrient tissue. Noteworthy also is the fact that the embryo is poorly developed in this family.
2. Numerous investigators agree that immediately following fertilization the endosperm grows much faster than the embryo. The quantitative data obtained in the present study amply confirm this fact. As an auxiliary to the embryo, therefore, the endosperm makes prompt and extraordinarily rapid development.
3. The collapse of seeds in alfalfa, which commences within 48 hours after fertilization, is associated with reduced rate of endosperm development. Little difference is apparent in growth of the embryo.
4. The first signs of abnormal development in seeds which abort following interspecific hybridization appear in the endosperm. There is considerable evidence on this point, but mention is made here only of the work of BOYES and THOMPSON (1) on *Triticum* and *Secale*. These investigators found early endosperm development much more different than embryo development after reciprocal interspecific hybridization, and observe that "Whatever may be the primary cause of poor seeds and lack of success in crossing, it expresses itself through the endosperm."

In the other group of seed plants, the gymnosperms, double fertilization does

not occur. Its innovation in angiosperms becomes understandable when the conditions under which the endosperm functions are examined. The endosperm in gymnosperms is derived from the megaspore without alteration of the cell lineage and completes its development as a haploid tissue. The seed is nourished by the parent sporophyte in both groups, but the circumstances under which this is effected differ. A brief consideration of certain features of the reproductive process in gymnosperms and angiosperms will help to bring into clearer focus the *raison d'être* of double fertilization in the flowering plants. Attention centers on the local provisions for nourishing the embryo.

The extensive mass of undifferentiated tissue in the female gametophyte of the more primitive gymnosperms—the cycads, for example—constitutes a large nutritive reserve which is immediately available to the zygote. In other words, abundant food for the young sporophyte is at hand at the time of fertilization. In the higher gymnosperms there is progressive simplification of the female gametophyte. More important than this for nutrition of the embryo, however, is the general tendency—which runs through the whole group—to mature the egg earlier and earlier in the ontogeny of the gametophyte. COULTER and CHAMBERLAIN (7) summarize the matter in the following words: “In the most primitive condition of the gametophyte, the eggs do not appear until the endosperm is nearly full grown; and other gametophytes can be selected and arranged in a series showing the gradual slipping back of the eggs in the gametophyte, until in such a form as *Torreya* the archegonium initial is differentiated as soon as wall formation has taken place.” This tendency reaches its climax in the angiosperms, in which the female gametophyte is reduced to a few cells, and the endosperm, as a definitive tissue, does not appear until after fertilization. One may logically infer that, in general, the endosperm of gymnosperms is sufficiently developed prior to fertilization to function immediately and directly in nourishment of the embryo. This mechanism might be expected to approach the limit of its effectiveness in the higher members of the group, owing to the great reduction of the gametophyte and the precocious differentiation of the egg. Although conclusive evidence is lacking, there are indications that this happens. The case of *Torreya taxifolia* may be cited as an example.

*Torreya* stands in an extreme position among gymnosperms in the tendency toward early development of the egg, although the endosperm consists of 400–800 cells at fertilization. In the mature seed of *T. taxifolia* is to be found a unique record of competition for the available nutrients between the developing endosperm and actively dividing cells just outside the megagametophyte. Ordinarily the endosperm in gymnosperms invades the surrounding tissue, the perisperm—more or less uniformly—consuming most of it. As described by COULTER and LAND (8), such invasion in *T. taxifolia* is extremely variable, leading to a highly irregular boundary between the two tissues in the mature seed. That the two

tissues are in competition is shown by the fact that growth in the one is in inverse relation to that in the other. The endosperm invades the tip of the nucellus, where the perisperm is not growing, obliterating all but a few peripheral layers of cells. Below the apical region, however, the perisperm is very active and resists invasion in many places. The greatest resistance is shown opposite the two vascular strands, where in the mature seed the endosperm is nearly cut in two by a deep constriction. The endosperm cells are radially elongated in regions of active invasion, and many of them are binucleate. Where the perisperm is less active, the endosperm cells are more nearly isodiametric and rarely binucleate.

There can be little doubt that in *Torreya*, proliferation of the perisperm restricts endosperm development. Carried somewhat further, the activity of the perisperm might entirely block endosperm development and thus deprive the embryo of nutrients necessary for growth. Sterility would then result. We have already shown that growth of the adjacent maternal tissues in angiosperms is a potential hazard to early endosperm and embryo development. The corresponding stages in *Torreya* seem not to have been studied, so that one cannot be sure that the phenomenon is homologous. The rather limited evidence from *Torreya* suggests that the endosperm as a strictly gametophytic structure nears its functional limits in the higher gymnosperms.

The relations discussed in the preceding paragraph may be looked at in a somewhat different way. COULTER and CHAMBERLAIN (7) emphasize that the endosperm is characteristically an aggressive tissue. Aggressiveness would appear to be a requisite for normal seed development, particularly where the young embryo is in competition with actively growing maternal tissue for a common nutrient supply. From this point of view, the margin which the endosperm must hold over the surrounding parts of the seed in order to insure nourishment of the embryo seems to approach the effective minimum in some of the higher gymnosperms. Further study of the early stages of seed development in the more advanced gymnosperms, such as *Gnetum* and *Ephedra*, would be of interest.

The angiosperms differ from the gymnosperms in three important factors affecting nourishment of the embryo: (1) The female gametophyte is reduced in size below that of the most advanced gymnosperms—in the typical case possessing only eight nuclei, one of which is the egg nucleus. (2) No endosperm is formed in angiosperms until after fertilization. (3) The angiosperm ovule contains little or no reserve food at the time of fertilization, in contrast with the considerable storage commonly occurring in the gymnosperms. As a result of these three circumstances the new sporophyte starts in an environment which might easily thwart its development. This follows from the fact that fertilization not only initiates development of the endosperm and embryo but also stimulates to active growth the previously quiescent cells of the surrounding maternal tissues. The lack of



reserves at fertilization and the non-appearance of storage products until the endosperm is well beyond the free nuclear stage mean that during this period the seed develops at a nutrient level which provides only for immediate tissue requirements. Translocated foods are used up as they are moved into the seed; there is no surplus. The much reduced gametophyte is small indeed relative to the total mass of the ovule. Originating at fertilization as simple uninucleate structures, embryo and endosperm must therefore compete for the limited nutrient supply with adjacent, well-established tissues of far greater extent. Starvation of the new sporophyte may thus easily occur if partition of nutrients within the seed gets out of balance. The success or failure of the young seed appears to hinge on the endosperm, as the nutritive agent of the embryo, establishing and maintaining a dominant position relative to the actively growing parts around it. Double fertilization, in the light of these considerations, emerges as a mechanism which enhances aggressiveness of the endosperm through the physiological advantages of the hybrid condition. It may also be thought of as a compensatory device tending to offset the disadvantage in reproduction associated with the extreme reduction of the female gametophyte in angiosperms.

The course of developmental events leading to seed collapse in alfalfa affords critical evidence in support of this interpretation. This type of seed failure, which we have called somatoplastic sterility (3), is attributed to inability of the endosperm to keep pace with growth in the surrounding tissues. Excessive meristematic activity in the latter results in starvation of the endosperm, and eventually abortion of the seed. The nutrient level immediately preceding and following syngamy is low, and probably the same foods are in concurrent demand by the protoplasts inside and outside the megagametophyte. The critical factor for survival appears to be the manner in which the nutrients are partitioned between the two regions. It is assumed that the available foods will be shared between the maternal tissues on the one hand and the endosperm and embryo on the other, in proportion to the velocity of growth in the respective tissues. If the rate of synthesis of nuclear material and cytoplasm in the endosperm falls below a certain minimum, which is frequently reached in alfalfa following self-fertilization, excessive growth is made in the inner integument or other adjacent maternal tissue, such as the nucellus, the endosperm is starved, and the seed collapses.

The occurrence of somatoplastic sterility reveals the delicate balance on which development of the seed in angiosperms hangs. Competition for nutrients may become acute between endosperm and maternal tissues. In order that the reproductive process be completed, a mechanism is demanded which will tip the scale in favor of the endosperm. Double fertilization is a method of conferring upon the endosperm the required advantage. It means that the endosperm of angiosperms

has two chances instead of one (as in the gymnosperms) of receiving the genetic equipment necessary to perform its function.

The differential survival of seeds in alfalfa following self- and cross-fertilization supports the hypothesis that the essence of double fertilization is a mechanism which secures to the endosperm the developmental advantages of hybridity.

The inequality of survival was determined by direct count of functioning and collapsing fertile ovules, the infertile ones being accurately separable. The increase in frequency of abortion after self-fertilization as compared with crossing within 144 hours after pollination was large, aggregating nearly fivefold. The difference characterized all seven plants tested. Since the two types of matings were made concurrently on each plant, the possibility of a factor other than the constitution of the male nuclei being responsible for the difference in survival is ruled out. One may think of the experiment as involving introduction into a common series of megagametophytes of the two classes of sperm as reagents. A reaction favorable to survival of the seed is evoked by one class of sperm (from an unrelated plant) in 93 per cent of the cases and by the second class (from the same plant) in only 66 per cent.

The reaction called forth by the two classes of sperm may next be considered. The rate of growth of embryo and endosperm derived respectively from the two products of fertilization was measured up to 6 days after pollination. Normal appearing proembryos and embryos are formed following both types of matings; if there is any difference in average rate of development at this stage it is too small to be established from the present data. The endosperm develops with great rapidity relative to the embryo, and it is in this tissue that a significant effect of cross-breeding is manifested. From the time of the first observations onward, the hybrid endosperm has a higher average number of nuclei than the inbred endosperm. Furthermore, the mean number of endosperm nuclei associated with the embryo from the zygote to the 4-celled true-embryo stage is regularly higher after hybridization. There is little reason to doubt that the added impetus to growth of the endosperm resulting from cross-fertilization is the cause of the higher survival. It may be supposed that a certain minimum rate of endosperm activity is essential to continued development of the seed; otherwise failure occurs through hyperplasia of the adjacent maternal tissues. Following self-fertilization, this rate of endosperm growth is attained in significantly fewer instances than following crossing.

The generally advantageous effects of cross-breeding on development of the organism are too well known to require more than brief mention. Heterosis is expressed in manifold ways, and apparently at any stage in ontogeny. It is well known in the mature endosperm through the work which has been done on maize. COLLINS and KEMPTON (4) found that hybridization with unrelated strains

increased the size of the ripe seed from 3 to 21 per cent in different *Zea* crosses. The existence of heterosis in the growing endosperm of alfalfa is demonstrated directly in the present investigation. The data are unique, so far as we are aware, in showing that hybrid vigor may be expressed as increased rate of nuclear division starting with a fertilization product.

LANDES (16) has shown that reduced seed formation in rye following self- as compared with cross-pollination is due, not only to a lower frequency of fertilization, but also to the collapse of fertile ovules. In these respects rye closely parallels alfalfa, as described earlier (3). LANDES found that abnormal development of the endosperm is common after self-fertilization. It seems probable, therefore, that seed collapse in rye, as in alfalfa, is associated with impaired capacity of the endosperm for growth. Heterosis in the endosperm following cross-breeding may be assumed to favor continued development of the rye seed, whereas the frequently occurring collapse of fertile ovules after selfing is best interpreted as an inbreeding depression effect.

The decreased rate of endosperm development in the self-fertilized alfalfa series is probably accounted for by the bringing together in homozygous condition of certain recessive genes with deleterious effects. KIRK (15) has shown that the vigor of the adult alfalfa plant is sharply reduced by self-fertilization, and the writers' observations on many families are in agreement. Defective individuals are also common in inbred families, such as seedlings with reduced chlorophyll production. It might be expected, therefore, that recessive genes adversely affecting the endosperm are also present. Since a single generation of self-fertilization reduces heterozygosity 50 per cent, the frequency of zygote and primary endosperm nuclei carrying such genes in homozygous condition is greatly increased in the self-pollinated series.

Aside from the fact that there are twice as many genes present, the nuclear condition to which inbreeding a diploid plant leads is like that prevailing in a haploid: the inhibitions resulting from dominance disappear, and the deleterious recessive genes exert their full physiological effects. In view of the sharp increase in failure of seeds in alfalfa accompanying a 50 per cent reduction in heterozygosity, it is apparent that fertility would be drastically impaired if the endosperm were haploid, as in gymnosperms.

The possibility is not excluded that partial self-incompatibility, per se, adversely affects seed development after self-fertilization. EAST (12) tested fifteen genes controlling self-sterility in *Nicotiana*, however, and found that none of them in homozygous condition weakened plant development. It is unlikely that fertile ovule survival is influenced by self-incompatibility as such.

It is necessary only to mention in passing the question whether the depressing effect of inbreeding on endosperm development in alfalfa is conditioned by a few genes

with conspicuous effects, many genes of minor influence, or both. MANGELSDORF (17) has identified a number of major genes in *Zea* causing defective endosperm. Those which give a viable plant in homozygous condition have markedly unfavorable action likewise on general development. In other words, these genes affect some basic physiological processes. Corn breeders recognize variation in endosperm development in highly selected inbred lines, which is probably attributable to the combined action of many genes with slight effects. It is likely that numerous genes, varying in magnitude of effect, are concerned with endosperm development in alfalfa.

Cross-fertilization, although not universal, is the prevailing mode of reproduction in flowering plants. EAST (11) and others have pointed out the broad evolutionary advantages it confers upon organisms as a concomitant of sex. The benefits of cross-breeding in relation to development have been demonstrated in many species, JONES' (14) work on corn being a classic example. Hybrid vigor in the endosperm, of course, cannot be a factor in seed formation in regularly self-pollinating species. If our interpretation of the role of the endosperm is correct, however, genes adversely affecting endosperm growth will tend to be automatically eliminated in autogamous forms.

In view of its generally invigorating effects, it is not surprising that hybridization should prove to be an important factor in completing certain processes ancillary to reproduction in flowering plants. Double fertilization, to be sure, is a novel means of extending the advantages of hybridity, but the end gained is clear enough. By this device the endosperm, playing a brief though vital role in development under unique circumstances, acquires a significant share in the benefits of sexuality.

### Summary

1. Following self-pollination in alfalfa, 34.4 per cent of the fertile ovules collapsed during the first 144 hours as compared with only 7.1 per cent after cross-pollination.
2. Fertile ovule collapse is associated with excessive growth of the inner integument. The first signs of abnormal development occur in the cells adjacent to the megagametophyte on the funicular side of the seed in the region of the vascular bundle. Breakdown of the endosperm follows, and the seed eventually aborts.
3. This type of seed failure is attributed to inability of the endosperm to keep pace with growth in the surrounding tissues of the seed.
4. The number of cells in the embryo up to 6 days after pollination increases in arithmetical progression. There is little difference in rate of growth following self- and cross-fertilization.
5. The number of nuclei in the endosperm increases exponentially during this period.

6. The rate of growth of the endosperm following hybridization is significantly higher than after self-fertilization.

7. The number of endosperm nuclei associated with an embryo at a given stage of development is regularly higher following cross- than following self-fertilization.

8. A survey of thirty-six species, in addition to alfalfa, shows that the angiosperm ovule is characteristically low in food reserves at fertilization. Early development of the seed is therefore dependent upon translocated foods. The endosperm is considered to play an essential role in the partition of these nutrients.

9. The evidence on differential survival of seeds following self- and cross-fertilization, growth of endosperm and embryo, histological changes associated with collapse, and comparative lack of ovule food reserves are considered in relation to the role of double fertilization in seed formation.

10. Conjugation of a male nucleus with the polar-fusion nucleus to form the primary endosperm nucleus is interpreted as a mechanism by which the physiological advantages of hybridity become available to the endosperm. Continued development of the young seed demands a rate of endosperm development which is attained more frequently following cross-fertilization by virtue of heterosis than following self-fertilization. Confirmation of this view is seen in the behavior of alfalfa seeds following the two types of matings.

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# NEW LEPIDOSTROBI FROM CENTRAL UNITED STATES

CONTRIBUTIONS FROM THE HULL BOTANICAL LABORATORY 514

G. B. MATHEWS

(WITH SEVEN FIGURES)

## Introduction

Knowledge of the structure of *Lepidostrobus* is based mainly on material discovered in Europe. The incrustations, of which many were described from the United States, furnish information concerning the external appearance of the organ or plant. Only three or four American species are recorded which were petrified and possible for anatomical study.

COULTER and LAND first described an American *Lepidostrobus* in 1911 (4, 5). The cones were found in Pottsville strata, near Indianola, Warren County, Iowa. In 1921 these investigators reported other cones from the same locality, but they could not expand the earlier description since the structure of the axis was not preserved in all the material. These specimens are now mainly in the collection of the Department of Botany at the University of Chicago, and are numbered: G21, P1-P11, and 502 (BF1, 21). JONGMANS (8, 9) named the cone described by COULTER and LAND *L. coulteri*. This *Lepidostrobus* is considered homosporous. In 1914 another species, *L. fischeri*, was described in detail by SCOTT and JEFFREY (13) from the Mississippian. It was later named *L. kentuckiensis* (14), since it was found in Kentucky, but the name which had been given it was preoccupied. In 1935 GRAHAM (6) described two kinds of small lepidostroboid cones from the Upper Connemaugh. These were found in coal balls from the Calhoun coal mine, Richland County, Illinois. Although these species are distinct from others hitherto described, the poor preservation of details led GRAHAM to leave the species unnamed.

It seems apparent that the cones upon which this study was made are of the same species as those described by COULTER and LAND (4, 5). The macroscopic features correspond well; the microscopic structures are the same, although COULTER and LAND could not give a description of the axis because it was not preserved. The cone with the best preservation is a cotype, G21 M1-M5. Another cone of which there are two sections in the collection is numbered G21 N1 and N2, and of the type specimen as described by COULTER and LAND (4) several slides have been made, G21 P1-P11. The variations in size of leaf traces as found in G21 M and G21 N and the differences in the structure of the lamina seem to be within the range of the same species.

*Lepidostrobos coulteri* Jongmans

## DIAGNOSIS

Cone large, 5-6 cm. in diameter; length up to 22 cm.

Axis 8-9 mm. in diameter.

Stele with central undifferentiated tissue; xylem 400-500  $\mu$  in thickness.

Leaf traces collateral; in outer portion of middle cortex 400-600  $\mu$  in diameter.

Ligule present; heel about 3 mm. long.

Parichnos present; bifurcates and branches, entering lamina.

Cortical layers about 3000-3400  $\mu$  thick.

Sporangium basal; palisade cells enlarged at corners.

Spores usually in tetrads of 52  $\mu$ , single spores 27  $\mu$ .

Lamina 12 mm. at base,  $\pm$  ridged, 24 mm. long.

Geological horizon: Pottsville.

The following description is based on five sections in the collection of the Department of Botany, University of Chicago, G 21, M1-M5. This previously undescribed specimen was found years ago with other cones in a coal pocket near Indianola, Iowa, the same locality from which the specimen described by COULTER and LAND came. The geological horizon is Pottsville.

This specimen represents only a portion of the entire cone. The five thin sections show the anatomical features fairly well. A radial section through the upper portion shows the stem axis with stele and cortex and the arrangement of the sporophylls (fig. 1A). Only a few remnants of the laminae can be seen. Of the other sections, one is median longitudinal (fig. 1C), showing arrangement and position of structures in and outside the stele. From it the spiral arrangement of the sporophylls, shape of the heel and lamina, and many microscopic structures can be seen. The other longitudinal sections are more or less parallel to the median one. One section is slightly oblique, passing from the outer cortex at the lower part through the middle cortex and almost into the inner cortex at the upper part. A section parallel to this last shows sporophylls, pedicels, and sporangia in oblique view at the upper part and the leaf traces in the outer cortex at the lower part. Another tangential section is through sporophylls which are at right and oblique angles.

The following data about the size of this cone are based on these sections, since no record was available as to the actual size of the entire cone. The longitudinal sections are at most 51 mm. long; with the upper end, which had been cut off, the entire fragment was about 7 cm. long. One side of the cone is not preserved, thus the diameter can be estimated only from the known radius, which is 4 cm. The diameter of the axis tapers from 9 to 8 mm., which suggests a rather large cone, probably not less than 20 cm. in length. The shape is cylindrical. Calcium car-



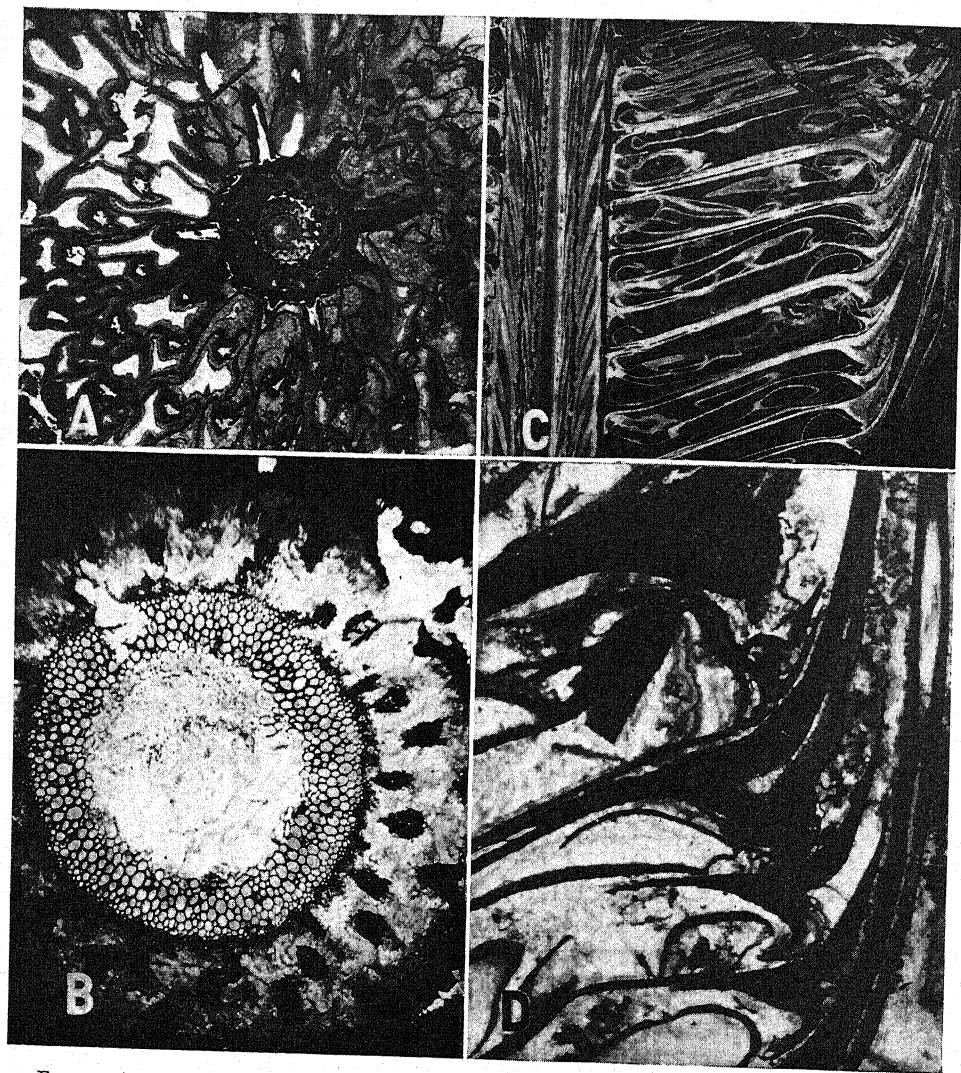


FIG. 1.—*A*: cross section of *Lepidostrobus coulteri* showing axis and sporophylls; G<sub>21</sub> M<sub>5</sub>. *B*: part of *A*. Central portion of axis showing stele with protoxylem and metaxylem, central tissue in upper part, and empty space between stele and inner cortex. *C*: longitudinal section showing axis with tissues and arrangement and structure of sporophylls; G<sub>21</sub> M<sub>1</sub>. *D*: part of *C* enlarged to show heel, parichnos, and leaf trace in lamina.

bonate was the main fossilizing agent. Other minerals, such as pyrites, were not noticed.

The lower and upper portions of the cone can be distinguished on the basis of several criteria: direction of the leaf traces in the axis, arrangement of the sporangia, relation between the pedicel and sporangium, location of the heel on the abaxial side of the pedicel, upward direction of the laminae, and other microscopic features. The spores were shed before fossilization, but here and there some are found in many sporangia. These spores are all of the same kind and apparently *in situ*.

#### CONE AXIS

The cone axis is cylindrical and has a diameter of 9 mm. at the lower end. It tapers a little to 8 mm. at the upper end in the distance of 51 mm. The radial and longitudinal sections show poor preservation of the central tissue, good preservation of metaxylem and protoxylem and of the inner and outer cortex, and fairly good preservation of the leaf traces in the stem axis. The core of the stele is not preserved except for some elements which are in contact with the metaxylem. These latter elements are of the same size as the mature metaxylem elements, 50–150  $\mu$  in diameter. All these cells have distorted cell walls, and of those most centrally located only the corners of the cells are preserved. The differences between these centrally located cells or “primitive fibers” and the metaxylem are that the former have little secondary wall thickenings and the end walls are at right angles to the longitudinal axis. While there are these slight differences, many similarities lead to the conclusion that these cells are to be regarded as undifferentiated metaxylem cells.

Adjacent to these central cells are the larger elements of the metaxylem. They have a diameter of 30–120  $\mu$ , while the thickness of the whole belt is 400–500  $\mu$ . The long section shows that there were eleven scalariform thickenings per 100  $\mu$  of length in the central part and that the number of thickenings increases peripherally to fourteen and twenty-two per 100  $\mu$ . The metaxylem elements gradually merge into the smaller elements of the protoxylem strand. The spiral thickenings of these latter are closer together, about thirty-six per 100  $\mu$ . The gradual transition is shown also by the decreasing diameter of the metaxylem elements and transitional forms of the thickenings. No longitudinal striae between the thickenings were noticed.

The cylinder of tissue just peripheral to the xylem is not well preserved. It is about 300–400  $\mu$  in thickness. A few remnants are present here and there, of small diameter (12–30  $\mu$ ) and parenchymatous. The space occupied by this layer is the one in which the endodermis and the pericycle might be differentiated, but these structures could not be clearly discerned. The leaf traces are preserved in this belt.

The cortex, which is differentiated into an inner, middle, and outer portion, thus resembles many forms already described. The inner cortex is bounded centrally by the inner space just mentioned. The crenulated outlines of the inner cortex are associated with the leaf traces, which extend through this tissue. A tangential gap occurs (fig. 1B), the total width therefore varying from 600 to 1000  $\mu$ . The polygonal cells are of various diameter, from 30 to 75  $\mu$ , with a length of 110  $\mu$ . From the longitudinal sections it is apparent that the cells of the inner cortex are not parallel to the strands in the stele in their longitudinal axes. The cells (or better, the cell walls of the inner cortex) gradually merge into the empty space peripheral to the inner cortex. The middle cortex, which was present where the space now occurs, is rather thick, almost 2 mm. No trace of any structure of the tissue formerly occupying this space could be detected. As in other forms, perhaps this trabecular tissue was too delicate to withstand the decaying influences before fossilization took place. From the rootlets and other structures of foreign origin present in the specimen, it is clear that fossilization took place a considerable time after the cone was detached from the tree. The parichnos, however, which extends from the middle cortex, may reveal something about the cellular peculiarities of this part of the cortex.

The leaf traces are well preserved as they extend through the middle cortex. The outermost portion of the stem axis is the outer cortex, a rather dark sclerenchymatous tissue 400–800  $\mu$  in thickness. Two kinds of cells can be distinguished. The inner ones are tangentially elongated and radially flattened, 25–30  $\mu \times$  12–15  $\mu$ . Several layers farther out the cells are isodiametric and spherical. In general the outer portion of this tissue is rather dark, and few details can be discerned. The lumen of these cells is 12–10  $\mu$ . This layer abuts the bases of the sporophylls, the pedicel.

The sporophyll is composed of the pedicel and the sporangium (fig. 1C). The maximal length for the pedicel up to the heel is 22 mm. The pedicel and with it the sporangium has a slightly upward direction, but the angle is not the same at both sides of the axis, owing to unilateral pressure. The pedicel is of triangular shape where it leaves the axis. For a short distance it is not connected with the sporangium, since no attachment of the sporangial tissue can be noticed and the epidermal layer is continuous around the pedicel. As the pedicel continues on the way out, it flattens and enlarges on both sides. Almost at the point where the sporangium ends, a strong downwardly projecting heel 2.5 mm. long is developed. The pedicel continues in a straight upward direction as lamina. The shape and size of the lamina are perceptible from several sections. The length is 24 mm., with a maximal width at the base of its origin of 12 mm. From the fact that each lamina shows a markedly enlarged portion on its section it is concluded that it had an abaxial ridge in which the vascular bundle was located. The blade of the lamina

is in some places  $150\ \mu$  thick, while the vein has a thickness of  $400\ \mu$ . The thickest lamina was about  $800\ \mu$  at a distance 8 mm. from the heel base. The pedicel thus enlarged from 1.3 mm. at the very beginning and 3 mm. at the base of the heel to a lamina 12 mm. in width and a total length of 46 mm.

#### PEDICEL

The heavy sclerized cortex is continuous with the pedicel. The sclerotic tissue surrounds the vascular bundle and the parichnos on all sides except in the region of attachment of the sporangium. In this latter area the adaxial epidermal and hypodermal layer is replaced by another tissue for almost the entire length of the sporangium. The tangential width of this connective tissue varies with the size of the pedicel at that special point. Usually it is in a relation of 2:5. From this tissue arise the sporangial wall, the tapetal tissue, and the subarchesporial pad. The heel has a peculiar structure. On the abaxial side no strong epidermal layer is present. The subepidermal layers have been destroyed up to  $400\ \mu$  into the tissue, and the empty space shows that this tissue was probably of delicate nature, although the epidermal layers show a wavelike contour over the heel. They assume the usual strong habit of dark epidermal and subepidermal tissue after 3-5 mm. The heel is filled with parenchymatous cells. The cells below the parichnos are of various size and extend at a right angle to the leaf trace but are arranged parallel to one another. The tissue which separates the leaf trace and the parichnos is more regularly arranged in vertical and horizontal rows several layers in thickness. The trapezoidal cells are  $50-80\ \mu$  long with a median diameter of  $25\ \mu$ . On the adaxial side of the heel the hypodermal layer is built up of rather long sclerenchymatous cells,  $300-400\ \mu$  long and  $20\ \mu$  broad. In the lamina the cells are isodiametric, especially those near the parichnos, and resemble cells of mesophyllous character. Small intercellular spaces are interposed among the cells adjacent to the parichnos.

#### SPORANGIUM

The sporangium is attached to the adaxial side of the pedicel and is 22 mm. long, 4 mm. high, and 5 mm. broad. The sporangia are mostly empty, although in many of them a few spores remained in the distal and proximal parts. By the flap at those parts pouches are formed which probably inhibited the spores from escaping. These spores are found in almost all parts of the cone, in the lower as well as in the upper section. The sporangia were ripe when fossilization took place, and the sporangia open, but some structures permit certain conclusions about the shape of the mature sporangium. On each lateral sporangial wall are two points where the cell walls are larger and functioned apparently for the stiffening of the wall. These enlarged palisade cells appear distinctly at almost the same loci in the cross-sectioned walls. They indicate the corners of the sporangial bag. The first thickening

is near the base at a distance which corresponds to the length of the wing of the pedicel. The next is about 1 mm. or more in front. After this follows the last portion, which is always a little longer than half the width of the relative pedicel at that point. Thus the mature sporangium would be of the usual shape, with a thickening on the four corners. The palisade cells are in general  $75\ \mu$  high. At the corners they reach  $125\ \mu$  but decrease toward the point of dehiscence to  $25\ \mu$ , while the diameter remains  $16\ \mu$ . The inside of the sporangium is lined with the tapetal layer, which is gone except in some instances at the distal or proximal pouches. These thin-walled elongated cells line the sporangium wall, sometimes eleven cells deep, to a total thickness of  $140\ \mu$ . The subarchesporial pad is composed of extremely delicate cells. No definite arrangement of sterile tissue plates was noticed.

#### SPORES

All except a few spores were shed. They are mainly single but in some instances are arranged in tetrads. Their surface is simple. The diameter of a tetrad is  $52\ \mu$ , while the largest diameter of a single spore is  $27\ \mu$ . The fact that they are of the same size and shape at the lower as at the upper part of the cone fragment leads to the conclusion that the cone was a staminate one or the plant homosporous.

#### LEAF TRACES

The arrangement of the leaf traces in the axis and sporophyll is visible in the long and cross sections (fig. 1A, B, C). The bundles take their origin from the points of the peripheral protoxylem. At the point of departure from the vascular cylinder there are about nine elements present, not surrounded by any preserved tissue. As the bundles go farther away from the stele, a surrounding sheath develops. These cells have a brownish appearance, while the xylem cells have a whitish crystalline content. The cells which compose the sheath are longitudinally elongated, with walls at right angles. The shape of the leaf trace in the inner space is radially elongated in cross section but becomes cylindrical as it gains distance from the stele. The number of xylem elements increases to about twenty-three but is not constant. The smaller protoxylem elements are usually in a mesarch position and number about seven.

With the approach of the leaf trace to the inner cortex, the appearance of a phloem portion in the trace becomes apparent. Where the leaf trace nears the inner cortex the tissue of the latter recedes, bulging away from the trace. At the same time the tissue which surrounds the trace sends out two lateral peripheral projections. These unite with the inner cortex, leaving between them an empty space which probably had been occupied by the phloem. A phloem strand in connection with the leaf trace cannot be seen in the inner space, probably because this belt was filled with parenchyma, which was not preserved. The vascular bundle

shows a different structure in its passage through the inner cortex. An empty space accompanies the leaf trace on its abaxial side. A sheath surrounds the bundle as a whole and the xylem strand which is separated from the phloem by the conjunctive tissue.

The delicate phloem tissue is not preserved at any place in this fragment—except for a small portion which probably had been broken off before fossilization began and thus had a chance to undergo mineralization by quick infiltration before decay started. The space occupied by the phloem in the inner cortex is cylindrical. Its tangential diameter decreases peripherally, while the radial one increases. Conjunctive tissue separates the xylem from the phloem. A different sheath surrounds the whole bundle as it passes through the middle cortex. This sheath is of the same structure as the tissue of the inner cortex. On the whole, except for size, the leaf trace in the middle cortex has the same arrangement and structure as in the inner cortex. In the middle cortex only the leaf trace is preserved, without a trace of the surrounding tissue. As in entering the inner cortex, the tissue of the outer cortex bulges back where the leaf trace approaches, but no projections of the sheath are formed. The clear phloem space in the bundle becomes flattened radially until it is crescent-shaped. A few remnants of cell walls on the periphery of the empty space indicate cells of small caliber. Another empty space appears in the outer cortex. It lies abaxial to the bundle as a continuation of the tissue of the middle cortex. In the same way that the phloem became apparent only in the next belt, the inner cortex, and the cortical bundle sheath in the middle cortex, so the parichnos shows up in the outer cortex, which is the following tissue. The soft delicate tissue is not preserved. The tangential width of the parichnos is the same as that of the whole bundle. The radial diameter is almost the same as that of the phloem. The total length of a leaf trace in the stem axis from the point where it leaves the protoxylem ring until it enters the pedicel varies from 1.5 to 2.5 cm.

These data are not taken from a single bundle, since none is cut longitudinally its whole length; they are based upon measurements of various leaf traces in different tissues of the cylinder. The course of the leaf trace is shown in the longitudinal axis. All leaf traces are cut in the same plane. On the upper end the traces are just leaving the inner cortex. A rather large portion is cut, about 3 mm. It runs parallel to the plane of cutting, while in the outer cortex the cut leaf trace measures almost 1 mm. in length, although enlarged during the journey through the cortical layers. The explanation is that the course of the leaf trace is rather steep in the beginning but becomes more horizontal in the outer half of the outer cortex. In the pedicel the position of the leaf trace is on the adaxial side close to the sporangium. Surrounding the leaf trace in the heel are the barred cells of the transfusion tissue on the adaxial side. These cells run not only parallel to the leaf trace but also at right angles to it. They accompany the leaf trace into the lamina and

are located laterally to it. There is no space left on the ventral or dorsal side because the trace fills out the space completely. An abaxial keel is formed which is rather prominent in comparison with the thin blade of the lateral wings of the lamina. At a distance of 22 mm. from the heel base, a leaf trace was noticed about  $300\ \mu$  thick. There three xylem strands could be seen surrounded by a mass of thin-walled elongated cells with oblique walls.

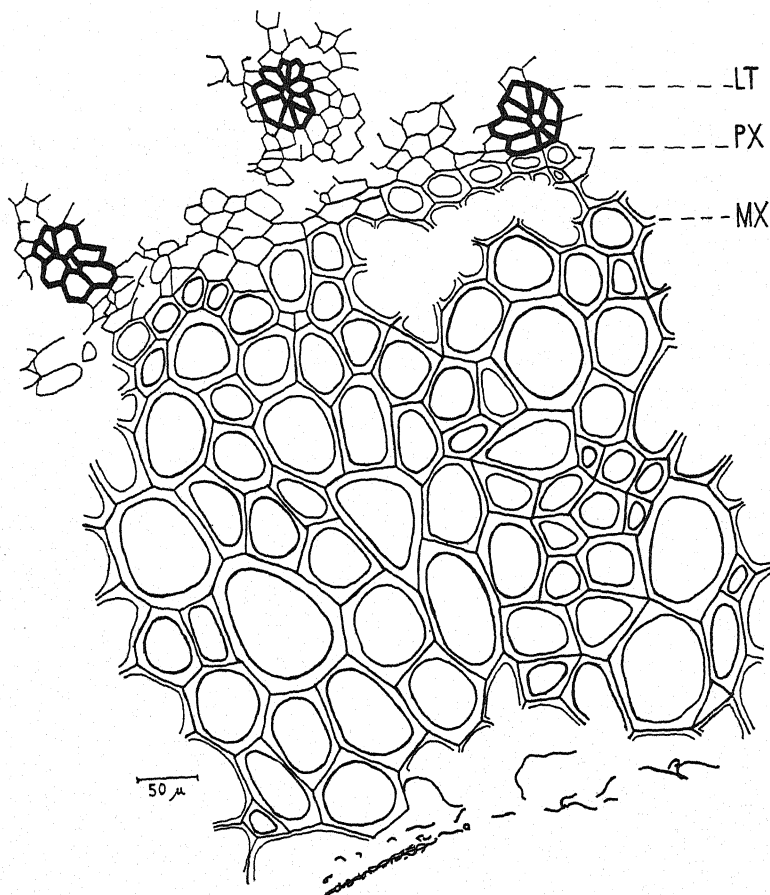


FIG. 2.—Axis of *Lepidostrobos coulteri* showing decayed central tissue, metaxylem, and protoxylem (*mx*, *px*), and some leaf traces (*lt*); G21 N1.

#### PARICHNOS

The parichnos extends from the middle cortex and is first noticed in the outer cortex on the phloem side of the leaf trace. It connects the lacunar tissue of the middle cortex with the intercellular spaces of the lamina. At about two-thirds the

length of the pedicel the parichnos splits off into three branches. The median one is at first bigger but soon decreases and after a short distance fades out, while the two lateral ones continue. In the heel the parichnos has a radial or tangential diameter of  $300\text{--}500\ \mu$  but decreases rather rapidly in width (fig. 1D). In the lamina it is surrounded by cells with intercellular spaces, two layers on the abaxial and one on the adaxial side. The space taken by the parichnos corresponds almost to the width of one cell layer of the surrounding tissue— $30\ \mu$ .

Two other mounted sections were found in the collection, G21 N1 and N2. One is a complete radial section; the other shows about a quarter of a radial section with the entire axis. These sections are (*fide* Dr. A. C. Noë) from the same locality as the first cone described. Both sections are no doubt from the same specimen. The external appearance suggests the same composition as that of G21 P, but in this specimen pyrite crystals fill the cells here and there.

The diameter of the cone is 6 cm., laminae included. The axis measures 8 mm.; the distance between the axis and the lamina is 2 cm. The central tissue is not preserved except for a few remnants. These are compressed and show as a rather sharp line separating the empty central space 1.3 mm. from the xylem, which is  $500\text{--}600\ \mu$  in thickness (fig. 2). The next zone is not preserved except for a few cells bordering on the protoxylem, and the leaf traces.

An approximate estimate can be given for the thickness of the space between xylem and inner cortex. From the preceding description it is evident that lateral projections extend peripherally from the leaf traces as they approach the inner cortex. The projections are preserved, the tissue of inner and middle cortex being destroyed. The space between xylem and inner cortex is about  $600\ \mu$  thick. The following space, occupied formerly by inner and middle cortex, measures  $2100\text{--}2400\ \mu$ . The leaf traces near the outer cortex measure  $400 \times 500\ \mu$ , with a phloem space of  $175 \times 75\ \mu$ . They are smaller than those of the previous cone, with  $600 \times 600\ \mu$  and a phloem space of  $200 \times 150\ \mu$ . The laminae are known only in cross section. The width of the lamina in the first peripheral row is 12 mm. Farther out in the third row some are  $1800 \times 500\ \mu$  and  $700 \times 300\ \mu$ . The middle vein is not sharp. The few spores measure  $25\ \mu$ . They are shrunk and attached to the sporangial wall.

### *Lepidostrobus noei* sp. nov. Mathews

#### DIAGNOSIS

*Lepidostrobus noei* Mathews, centimeter 15 longus, 7 crassus, bracteis millimeter 8–20 longis, laminis medio-costatis millimeter 14 longis, 8 latis; sporis dimorphis magnitudine comparatis ratione 1:5, microsporis minutis quaternatim conjunctis millimeter 0.050; macrosporis millimeter 0.275–0.330 innumerabilibus sporangia inferioris tenentibus.



Cone large, heterosporous, 7 cm. in diameter and more than 15 cm. long (actual length of fragment 11 cm.).

Axis without central tissue, 7 mm.

Stele with central tissue of undifferentiated xylem; xylem layer 300-400  $\mu$  thick.

Phloem surrounding the xylem.

Pericyclic fibers present.

Leaf trace collateral; xylem elements more than thirty; diameter of leaf trace (with slight variation) 100  $\mu$  in axis.

Parichnos not extending into peripheral part of outer cortex.

Sporangia basal, five-sided polygon, sterile plates short, unbranched.

No heel present.

Megaspores numerous in sporangia at base of cones, smooth, with triradiate ridge, 275-330  $\mu$ .

Microspores in sporangia at top of cone, tetrads 76  $\mu$ , single 50  $\mu$ .

Laminae 8 mm. broad, with sharp vein; 14 mm. long.

Geological horizon: Upper Devonian (?).

The new cone is named after the late Dr. A. C. No .

This cone was found near Gartersville, Gorrard County, Kentucky. It was forwarded to Dr. A. C. McFARLAN of the Department of Geology at the University of Kentucky, Lexington. Dr. A. C. No , University of Chicago, to whom the cone was sent for classification, gave it to the writer for a superficial study. When it was found that the cone was heterosporous, all efforts were made by Dr. No  to get permission from the respective authorities in Lexington to section the cone and make a detailed study. The sections and slides are now in the Department of Botany at the University of Chicago, nos. G22 M1-M33. A big fragment showing a long section and partial radial section with the external structure intact is now in the Department of Geology, University of Kentucky, no. M3121. As to the geological horizon, Dr. McFARLAN wrote that the cone was found "on the edge of the Knobs, which would make possible its occurrence in either Ohio or New Providence shales. It is unfortunate that the precise horizon is not known." The Ohio shale would place the cone in the Upper Devonian; the New Providence shale, in the Mississippian. The New Providence shales contain only marine fossils, as is known until now. Before any sections were made, some excellent molds were made in plaster of paris by the courtesy of the Daprato Statuary Company, Chicago, Illinois.

#### GENERAL DESCRIPTION OF CONE

The cone is a petrified specimen and is almost free from adherent foreign material, so that the external structure can be seen. The color on the outside is gray and inside, brownish. At the lower part a greenish taint was caused by the pres-

ence of algae and protonemata of mosses. The fructification was exposed to climatic influences for considerable time. On the lower part a few sporophylls were broken off. On one side the laminae are not present for almost the entire length of the cone. Fossilization of the cone is no doubt excellent; the mineral, however, is mainly calcium carbonate and is decayed to a large extent. Peels do not therefore reveal the details. The observations and pictures were made mostly with reflected light. Some of the megaspores were replaced by siliceous compounds, others by pyrites. The connective tissue between pedicel and sporangium is not preserved but is replaced by transparent calcites.

The length of the fragment is 11 cm. (fig. 3*B*). The maximal width is 7 cm., about 8 cm. from the lower end. The cone is wider at the middle, tapering toward the top and bottom. By pressure of unknown forces the cone—and with it the axis—was deformed from a cylindrical to an elliptical shape, so that the diameter varies. The small diameter is 5.5 cm.; the large one is 7 cm. Compression occurred in such manner that on one side the laminae were appressed and the cone broke longitudinally. The halves were disarranged, so that on the same radial section the cone axis on one side measures 2.5 cm. in thickness (except the central tissue) and on the other side 3.1 cm. The weaker central tissue is mostly crushed. All laminae have been broken just at the point where one would look for the ligule. Where the laminae had been broken off before fossilization, decaying processes have exposed spores and the structure of the pedicels. The spores could be seen with the naked eye and filled the sporangia in great masses; surely megaspores. A small piece of a broken sporangium from the upper end contained spores much smaller than the megaspores in the sporangia below. In the same uppermost whorl were also some megasporangia; thus this whorl consisted of mega- and microsporophylls.

An upper part, 1.8 cm. in thickness, was cut off radially to study the arrangement of mega- and microsporophylls, which seemed to be unusual. The remaining lower portion was sectioned tangentially near the axis. Another radial section was then made, since the structure of the axis was found not to be well preserved in the uppermost portion. Other sections were cut parallel to the longitudinal sections at 4 mm. intervals. In these sections the details of all organs were exposed.

The general arrangement of the parts in this cone is very similar to that described from other strobili of this genus. The stele is surrounded by a well-preserved parenchymatous layer. The cortex is composed of three layers, exceptionally well-preserved tissues. The outer cortex is continuous with that of the pedicel. The sporangium is attached to the adaxial side of the pedicel for almost its entire length. The pedicel does not develop a heel but bends up, becoming the lamina which overlaps several sporophylls (fig. 3*A*). The megasporophylls are found in the lower part of the cone and the microsporophylls in the upper part. The axis tapers gradually toward the upper end, but the shape of the cone does not corre-

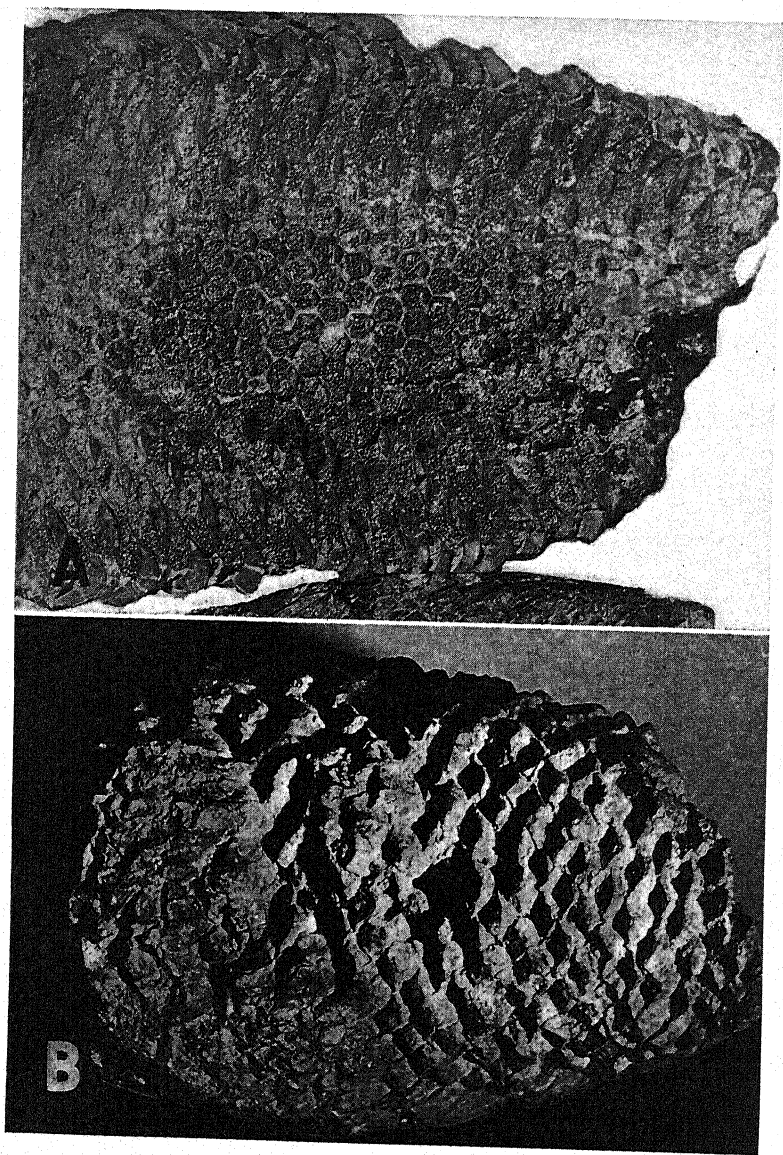


FIG. 3.—A: longitudinal tangential section of *Lepidostrobus noei* showing arrangement of megasporophylls and laminae; M<sub>312r</sub>. B: external view.

spond to this feature of the axis. From the axis the conclusion may be drawn that the cone was about 15 cm. long. Thus in the lower two-thirds are megasporophylls with microsporophylls in the upper third.

#### CONE AXIS

The central portion of the stele consists of cells which are considered to be undifferentiated xylem elements. Not all are preserved. Those preserved show that they are of the same size and shape—except for the thinner walls and lack of secondary thickenings—as the adjacent metaxylem elements. Owing to the crushing of these cells their exact shape can be determined only by comparison with metaxylem elements. The central cells have no content, are rather long, and their cross-walls are pointed or at right angles to the longitudinal axis. The name pith cannot be applied to this tissue. The xylem is continuous with the central tissue and has a centripetal development, with the protoxylem elements on the outer periphery.

The following measurements are given from the side of the shorter diameter. The metaxylem belt is eight to twelve cells deep, with a total thickness of 300–400  $\mu$ . The elements vary from 50 to 130  $\mu$  in diameter and have scalariform thickenings. The larger elements are filled with a centrally located substance an average distance of 12  $\mu$  from the wall. Elements of less than 25  $\mu$  therefore do not have this infiltration. The middle lamella of the cell walls shows up clearly as a black line between the light brown walls. The structure of the stele is exarch, the protoxylem elements being located at the periphery of the stele. These protoxylem elements are smaller, about 20  $\mu$  in diameter. The abaxial circumference of the xylem belt is regularly pointed by protuberances of the protoxylem. These are the beginning points of the leaf traces. The protoxylem abuts on a cylinder of parenchymatous cells. It is this layer which in most of the cones described is poorly or not at all preserved. In this cone, however, the preservation is excellent. A camera lucida drawing was made of an area of this parenchymatous region to show all possible details (fig. 4).

The protoxylem is bordered by a zone of two to six tangentially elongated cells measuring about 25  $\mu$  in length and 8  $\mu$  in width. The walls are thin, the black corners show up sharply, and no intercellular spaces are seen. This zone is interrupted where a leaf trace is to be given off and is several layers in thickness where a leaf trace is to emerge. It resembles the phloem of the leaf traces and represents, by its position and anatomical character, tissue which can be called phloem. Just peripheral to this cylinder is another belt of parenchymatous tissue containing numerous leaf traces. These two parenchymatous cylinders are not clearly delimited from each other. The outer limit of the peripheral layer can be identified by a peculiar layer of cells. They are thin-walled, elongated tangentially, and their radial walls are here and there crushed and zigzag in shape. In connection with the

cells in the pericycle are found one or more cells with thick walls representing pericyclic fibers (fig. 4 $pf$ ). The innermost layer of the cortex consists of cells of larger diameter (40–70  $\mu$ ) with a central heterogeneous filling. These cells abut peripherally on a trabecular tissue, which is well preserved. This belt measures 400–800  $\mu$  in diameter. The elongated cells range in length from 30 to 200  $\mu$ , and run in various directions. The cells on the inner border of this belt usually begin with a smaller diameter than that of the adjacent cells, but enlarge from 6 to 30  $\mu$ . Even in the same cell the diameter varies, giving this tissue an irregular appearance.

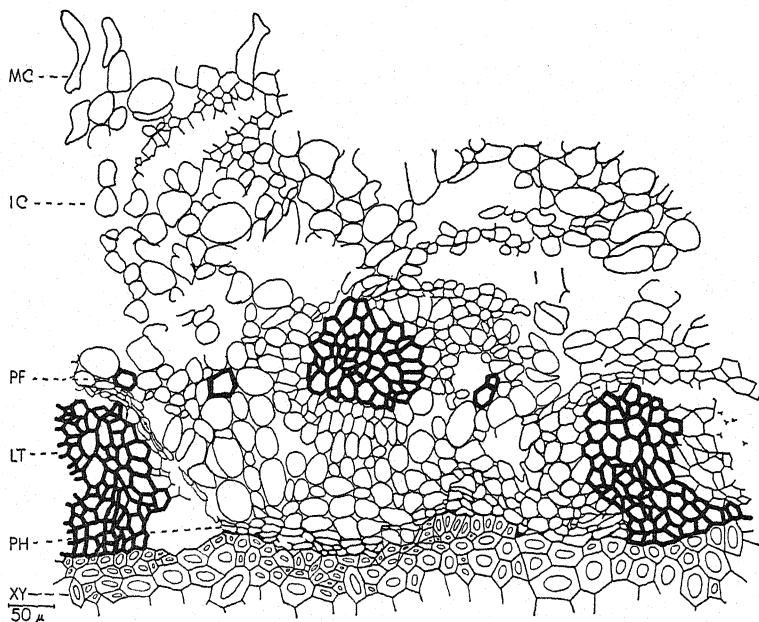


FIG. 4.—Part of axis of *Lepidostrobus noei* (xy, xylem; ph, phloem; lt, leaf trace; pf, pericyclic fibers; ic, inner cortex; mc, middle cortex).

The aerenchymatous nature of this tissue is apparent. The outer cortex starts abruptly and is 1100–1200  $\mu$  in thickness. It is made up of two kinds of cells, in two concentric layers. The cells of the inner ring gradually merge into the outer tangential belt. In some instances an irregular black line separates these layers. Among the outer cortex cells of the inner portion intercellular spaces are interspersed, and the diameter of the cells varies a little. On the whole they have a homogeneous content. Although a sharp line cannot be drawn between the peripheral and the central portion, a difference is indicated by the different structure and arrangement of the cells. The cells of the outer portion are arranged in more or less clear radial rows and the diameter decreases peripherally to 23  $\mu$ , especially where a pedicel is to leave the axis.

## SPOROPHYLLS

The sporophylls branch off from the axis at almost right angles, but the inclination of the sporophylls with the axis varies. In the lower portion it is at  $70^{\circ}$ ; at the upper part, at  $80^{\circ}$ . The sporophylls are arranged in spirals. The phyllotaxy is 2:51. Some indication of this arrangement may be noticed in the regular appearance of the lamina and sporophylls in the long section (fig. 3A). The sporophyll is composed of the basal pedicel and the upper sporangium. The pedicel is continuous with the outer cortical tissue of the axis and has cells of the same sclerenchymatous structure. The connective tissue between pedicel and sporangium is in no case preserved, but from the absence of the sporangial wall on the abaxial side of the sporangium it is apparent that the latter was attached to the pedicel for almost its entire length—except for a small distance on the proximal and distal ends. The fragment of the cone represents the lower portion. The lowermost sporophyll is 7 mm. in length, the uppermost 22 mm. When at first the pedicel becomes free from the axis it is triangular in cross section and 2 mm. in the longest diameter. The horizontal diameter is always smaller than the overlapping sporangium, except at the distal end. Here the pedicel enlarges laterally to 5 mm. As the sporangium tapers at the periphery of the cone the pedicel enlarges and becomes rounded on its abaxial side. The cells of the pedicel are of the same structure as those of the outer cortex, isodiametric, sclerenchymatous. The leaf trace runs on the adaxial side. The epidermal cells have thick walls and are arranged in one layer. No difference was noticed between pedicels of mega- and microsporophylls.

## SPORANGIUM

The sporangium is attached to the adaxial side of the pedicel. Both mega- and microsporangia are found in *L. noei*. The structure is the same, except the spores. The shape of a sporangium in cross section is shown in figs. 3A, 5B. It measures  $2 \times 2.2$  mm. By this shape all sporophylls match perfectly. The sporangium is slightly larger than the pedicel, except at the distal end, where the sporangium flattens and tapers tangentially. The connective tissue forms a rather narrow band and is about one-fifth the total width of the pedicel. The walls of both the mega- and microsporangia are made up of typical palisade cells,  $25 \times 25 \mu$  in size. No difference was noticed in the size of these cells at the corners or on the tip of the sporangia. Inside these wall cells is another layer of jacket tissue made up of elongated cells in which a dark spot (nucleus?) can be seen. In the palisade cells a dark spot is located at the outer periphery (fig. 5D). The tapetum centers the jacket cells. It is conspicuous all round the sporangium but is especially well developed at the corners, with a depth of eight to eleven cells. These cells are continuous with the subarchesporial pad. All sporangia are in the mature stage and not yet opened. About the mechanism of dehiscence no peculiar structure could

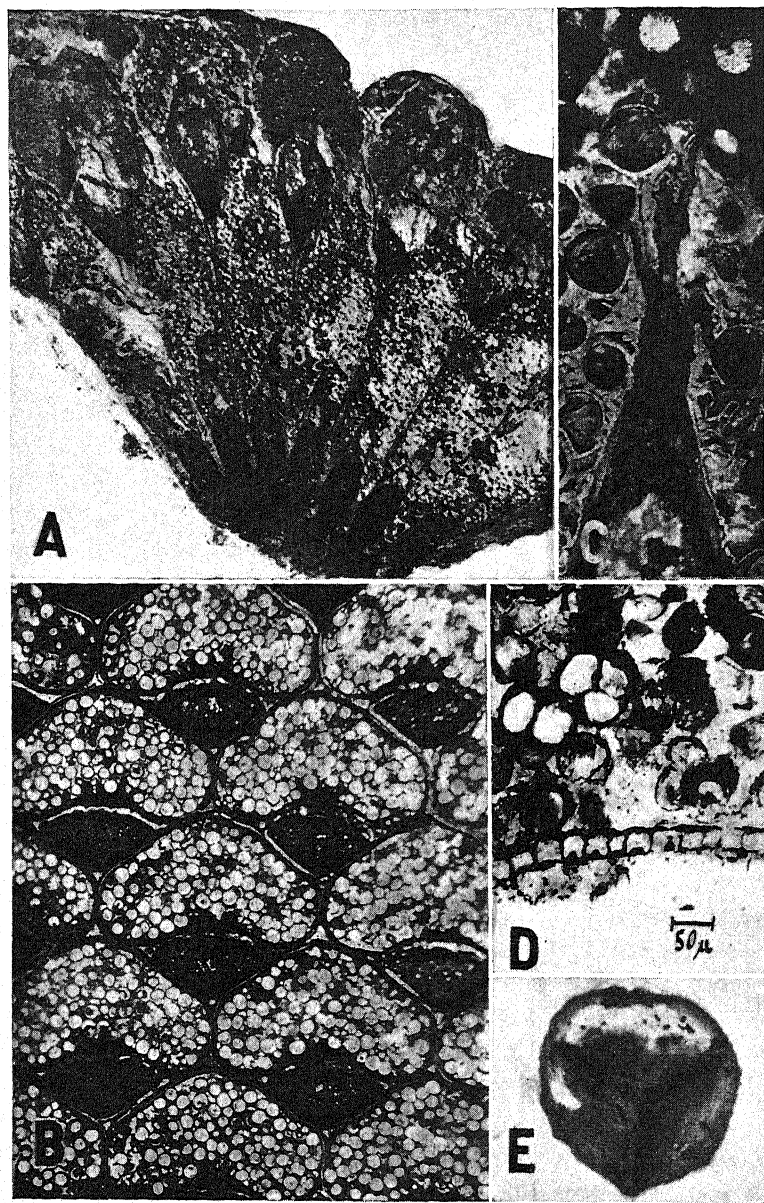
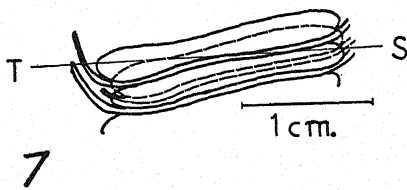
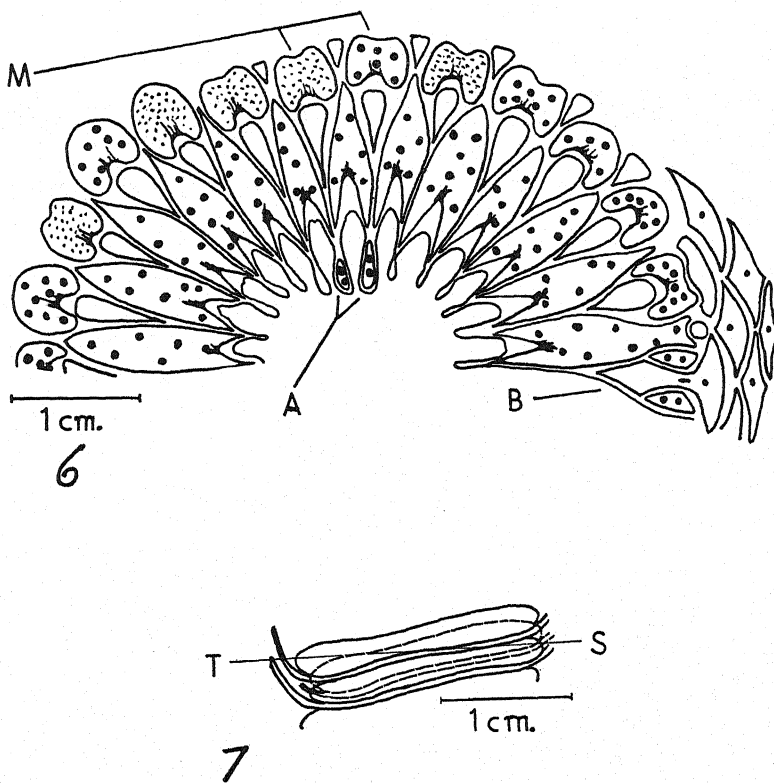


FIG. 5.—*Lepidostrobus noei*. *A*: cross section of cone showing arrangement of micro- and megasporophylls; *B*: tangential section showing megasporophylls; *C*: oblique section through megasporophyll showing sterile tissue plate and megaspores; *D*: microsporangium with microspores in tetrads and sporangial wall; *E*: megaspore.

be noticed in the wall. Sterile plates arise from the subarchesporial pad in both the mega- and microsporangia (fig. 5C). In more oblique sections these plates are finger-like projections.

#### ARRANGEMENT OF SPOROPHYLLS

The arrangement of the mega- and microsporophylls is peculiar. Figure 6 represents a drawing from a photograph of a radial section through the upper part of



FIGS. 6, 7.—*Lepidostrobus noei*. Fig. 6, arrangement of micro- and megasporophylls. Fig. 7, diagram showing plane of section as given in fig. 6.

the cone. It shows in the peripheral layer thirteen sporophylls—eight megasporophylls and five microsporophylls. The microsporophylls originate separately at three different loci in the same spiral. The median circle represents only megasporangia; the inner circle shows in two instances megasporangia (fig. 6A). It would seem that these sporangia are the same as are cut again at the outer circle, which would mean that the same sporangium (A and M) has megasporangia and microspores. From a longitudinal section, however, the solution of this striking feature can be explained in another way. As shown in figure 7, three whorls are



cut longitudinally, of which the upper and lower sporophylls are on the same orthostichy; the median is interlocking. If the upper sporophyll is a microsporophyll and the lower ones are megasporophylls, it is apparent that by a radial section (as indicated by the line *T-S*) the arrangement of sporophylls will be a microsporophyll at the outer circle and a megasporophyll at the median and inner circle. Figure 6 shows various stages of the relation between pedicel and sporophyll. At various points the sporangium overlaps the pedicel, and at *B* the sporophyll is cut in such a way that the pedicel is seen to be continuous with the lamina, while small lateral portions of the overlapping sporangium have been cut off. The pedicels have the same structure in both kinds of sporophylls, although it seems (fig. 6) that the pedicels of the microsporangia are smaller than are those of the megasporangia.

#### SPORES

The megasporangia are filled with numerous megaspores. Some are wholly replaced by pyrites, siliceous compounds, or calcites. After maceration of some megaspores with HCl the thin spore wall remained, which made a detailed study of the spore wall possible. The megaspores measure 270–330  $\mu$  and show triradiate markings, each about 120  $\mu$  long. The megaspores are separate. Here and there crushed megaspores are seen. The microsporangia are in the upper part of the cone. From a reconstruction on the basis of the preserved axis apparently the upper third of the cone had microsporangia. The microspores are arranged in tetrads (fig. 5*D*). The diameter of a tetrad is 76  $\mu$ ; of a single spore, about 50  $\mu$  in the longer axis. In sections of the microspores often exosporium and endosporium can be seen.

#### LAMINA AND LIGULE

As previously mentioned, the laminae are broken at the point where one would look for the ligule (fig. 3*A*). It was not possible to locate with certainty a structure which might be considered a ligule. The lamina starts with a constriction of the pedicel. It is at that point cylindrical or triangular in cross section, with a diameter of 3.5–4 mm. Soon the lamina widens tangentially to 8 mm. On the surface of the cone where the laminae are preserved a small longitudinal median ridge can be seen on each lamina. The longest lamina was 14 mm.

#### LEAF TRACES

The leaf traces are well preserved. The starting points are protoxylem elements on the periphery of the stele (fig. 4). The number of such elements is about ten when the leaf trace is freed from the xylem belt. The trace is 100  $\mu$  in diameter. The smaller elements are in the center and probably represent the protoxylem. As the leaf trace passes into the parenchymatous layers, the cells adjacent to the bundle are arranged around it like a sheath. In this parenchymatous belt more leaf

traces can be seen than in the regions which follow peripherally. On the abaxial side of the leaf trace a few small, thin-walled cells are separated from the xylem by a layer of parenchymatous cells which represent the beginning of the phloem strand. The leaf trace does not enlarge much as it passes through the trabecular tissue of the middle cortex. In the outer cortex very few leaf traces are seen. Near the middle cortex these traces may be surrounded on all sides with trabecular tissue, although a layer five or more cells in thickness may separate the trace and the middle cortex. A parichnos strand was not noticed in the peripheral part of the cortex or the pedicel. At the entrance of the leaf trace into the pedicel the diameter of the trace is not increased. When the trace bends in the outer cortex to enter the pedicel, the arrangement of the cells outside the bundle on the phloem side is changed. The sclerenchymatous cells on the adaxial side run in their ordinary straight way and simply abut on the bundle sheath; the cell on the abaxial side bend to enter the pedicel. Just at this point parenchymatous cells parallel to the main axis are seen which connect the leaf trace with sclerenchymatous cells. This layer is about  $300\ \mu$  in thickness. In the pedicel the leaf trace runs on the adaxial side, phloem downward. It is larger than in the axis and has a radial diameter of  $200 \times 500\ \mu$ , of which the latter means the axis in the xylem-phloem direction. In the lamina the leaf trace takes a central position, is of large diameter ( $400 \times 500\ \mu$ ), and collateral. The epidermis is composed of a layer of thick-walled cells.

### Discussion

A comparison of *Lepidostrobus coulteri* and *L. noei* with the already described species of *Lepidostrobus* does not reveal many striking peculiarities in these new cones, but they appear to be good distinct species.

In size the cones agree with *L. brownii* (17), which has a diameter of 5-7.5 cm., and with *L. kentuckiensis* (13) and *L. bertrandi* (16), which have diameters of 5 cm. Data of the actual length of the cones are not always available, for generally only fragments are known. The longest in this group is *L. coulteri*, with an actual length of 22 cm. (5).

The axis varies much in size and structure in this genus. The ratio between the diameter of cone and axis is not constant: in *L. brownii* (17) it has a range of from four to ten, in *L. coulteri* and *L. delagei* (17) about seven, in *L. oldhamius* (11) five to ten, in *L. noei* about ten, in *L. bertrandi* (16) four, in *L. kentuckiensis* (13) five, and in *L. binneyanus* (1) three. In *L. masleni* (3) the axis decreases from 1.28 mm. at the base of the cone to 0.53 mm. at the top. The diameter decreases in the same ratio. This is not true in *L. noei*.

According to the structure of the stele the cones may be placed into three groups. To the first group belong the protostelic forms with xylem throughout, such as *L. schimperi* (17). In a second group can be placed those forms which have

not developed a special pith tissue but have central undifferentiated embryonic tissue. Here belong the forms described as having "fibres primitives," as *L. brownii* (17), *L. kentuckiensis* (13), and *L. bertrandi* (16). In this group fit also *L. coulteri* and *L. noei*. To the third group belong the forms with a central pith—the siphonostelic cones, as *L. binneyanus* (1), *L. masleni* (3), and *L. arberi* (1). From the phylogenetic standpoint, considering only this character, the first group probably is the most primitive while the last one with a pith is most advanced. The forms in the second group bridge the other two groups.

In the central embryonic cells of *L. coulteri* and *L. noei* are found thin walls and walls with thin scalariform thickenings, pointed cells with oblique cross-walls, and cells with end walls at right angles. In all forms the scalariform elements are central, while spiral or annular thickenings are found in the peripheral protoxylem elements. The nature of the region just outside the xylem cylinder is much disputed. This is due not only to insufficient preservation in most forms, but partly to a real difference in the arrangement of tissues in different cones and partly to a lack of uniformity of nomenclature. The tissue surrounding the xylem belt was probably of delicate nature. If it did not decay it became crushed and only in a few cases is it well preserved. These small cells of a parenchymatous nature were called cambial cells in *Lepidodendron* by WEISS. SEWARD and CAMPOS (3) consider them meristematic. BERTRAND described them as "fibres primitives" and considered them related to phloem cells. MASLEN (11) speaks of them simply as parenchymatous cells. BOWER (2) did not recognize any phloem, although the specimen he described was well preserved. SCOTT (15) calls them phloem cells. In *L. noei* the tissue (fig. 4ph) consists at certain points of several layers. The name phloem seems to be appropriate. Hypertrophied cells with darker contents are described by BERTRAND and SEWARD as secretory cells. Polygonal thick-walled cells interspersed at regular intervals are figured by BOWER (2). They have not been recorded from other cones, and according to position and anatomy they are described as pericyclic fibers. Barred cells, occurring on the outer periphery of the xylem, have been observed in *L. oldhamius* (11) and more rarely in *L. bertrandi* (16) but have not been noticed in the other forms here mentioned.

The endodermis, which in recent pteridophytes is markedly differentiated from the other cortical and pericyclic layers, was described and figured by BOWER (2) and ZEILLER (17) for *L. brownii*. BOWER recognized an endodermis "by the more sharp definition of its walls . . . and the radial walls with characteristic dots" (2). The endodermis BOWER observed was not continuous. ZEILLER indicated tentatively as endodermis a cell layer characterized by distorted zigzag radial walls. He could not follow this sheath around because in many instances the

tissue was decayed (17, pl. II, fig. 8). This wall deformation was the only anatomical character; no wall thickenings or dots were noticed.

Division of the cortex into three parts is in general use. The inner cortex is usually preserved, but if not, the beginning of the internal periphery can be made out by the abaxial projections of the leaf trace, as is seen also in *L. bertrandi* (16) and *L. oldhamius* (1). The nature of the middle cortex as an aerenchymatous tissue, as in *Selaginella*, is well shown in *L. noei*. The lacunar tissue is probably easier attacked and destroyed by decay organisms than the other tissues. The tissue of the middle cortex is continuous into the leaves and laminae as a small strand on the phloem side of the bundle, the parichnos. In different species the development of the parichnos shows in various degrees. In *L. coulteri*, as in other species, it branches at the distal end of the pedicel and accompanies the leaf trace on both sides for a certain distance; in *L. brownii* (17) it terminates in the outer cortex; in *L. noei* almost the same is true; in other forms, as *L. bertrandi* (16), no parichnos is seen at all. The presence of the parichnos seems to be specific. It is not impossible that the degree of its development may reflect the ecological habitat of the specimen.

The sporangia are usually the shape of a five-sided polygon. The sporangium is attached basally to the adaxial side of the pedicel in most forms, or it is median—as in *L. masleni* (3)—at a distance of 1 cm. from the base of the pedicel. The sporangia of *L. noei* are quite long, reaching 20 mm. and being exceeded only by those of *L. masleni* (3), which attain a length of 27 mm. The jacket layer, consisting of an inner and an outer palisade layer, is well differentiated from the tapetal layer in *L. noei*. Owing to early development, the subarchesporial pad extends farther into the sporangium, but later becomes appressed to the floor of the latter, as can be seen by the compressed cells. Probably the growing spores are responsible for this.

Sterile tissue plates have been noticed in *L. oldhamius* and *L. masleni* (in the microsporangia), and their structure is specific. SCOTT (15, Vol. II, p. 165) mentions evidence of sterile tissue in megasporangia but did not illustrate his statement. *L. noei* shows sterile tissue plates also in the megasporangia (fig. 5C). Special enlarged palisade cells at certain points have been described in several forms (such as *L. brownii* and *L. coulteri*). About the function there is no agreement. Two species are known in which the number of megasporangia in a sporangium is greater than sixty-four, *L. brownii* (17) and *L. noei*. A comparison of the size of micro- and megasporangia within a species shows that the ratio in the homosporous species is 1:1, but varies in the heterosporous species. In *L. bertrandi* it is 1:2; in *L. noei*, 1:5; in *L. brownii*, 1:10; in *L. scottii*, 1:30-40, and in *L. masleni*, 1:45. Phylogenetically *L. masleni* would be considered in this respect highly specialized

and *L. bertrandi* less advanced. Thus a cone may be highly specialized in one respect and primitive in another. The presence of the heel seems to be specific. It is present in most forms but is lacking in *L. noei*. The presence of a ligule is specific for *Lepidostrobus*. It may be inserted in a ligular pit as in *L. arberi* (1) or occur without a ligular chamber.

The structure of the leaf trace is the same in various forms. It is collateral except in *L. oldhamius* (1), where it appears to be concentric at the apex of the sporophyll. The number of xylem elements in the leaf trace varies among the species and may range from nine to as many as seventy. In traversing the axial tissues the leaf trace is accompanied in a peripheral direction by each of the tissue systems through which it passes; for example, a trace passing through the middle cortex is accompanied by phloem, parenchyma, and inner cortical tissue in the immediately peripheral layer. Thus xylem, phloem, parenchyma, etc. are found displaced in a peripheral direction immediately adjacent to the leaf trace.

The sporophylls are arranged in circles or spirals. The data given by ZEILLER (17) show that the phyllotaxy is not constant in *L. brownii*, the number of orthostichies varying from seventeen to thirty-three. In *L. noei* the number is even larger, fifty-one. The partly irregular arrangement of mega- and microsporophylls in *L. noei* seems to be unique. Only by study of additional heterosporous forms can it be determined whether this arrangement of sporophylls is common.

The epidermis is composed usually of thick-walled cells. Some species show a special hypodermal layer.

### Summary

1. Two species of *Lepidostrobus* are described, *L. coulteri* Jongmans and a new species, *L. noei* Mathews from Kentucky. Two specimens of the former are described in detail, since nothing was known previously about the structure of the axis of that species. A description and several diagnoses are made on the basis of sections. The size, the presence of a heel, the extension of the leaf traces high into the lamina, together with a well-developed parichnos seem to distinguish these specimens from other described cones. The five cones in the collection suggest a classification among homosporous *Lepidostrobi*.

2. *L. noei*, sp. nov., is unique in that it shows many features not yet observed in other forms. The middle cortex shows the trabeculate structure quite well, although the parichnos does not extend beyond the central portion of the outer cortex. The heel is absent. The cone is heterosporous, the microsporophylls occupying the upper one-third of the cone and the megasporophylls the lower two-thirds. No difference in structure was noticed between micro- and megasporophylls. The ratio in size of the spores is 1:5. The arrangement of micro- and megasporophylls in the same whorl is unusual.

3. A comparison with the hitherto described cones has been made and differences and similarities pointed out. Phylogenetic considerations have been contrasted with the geological appearance of the cones.

The writer is indebted to the late Dr. A. C. NOÉ for suggesting this problem and supplying the material.

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## PEPTIDASE ACTIVITY IN THE AVENA COLEOPTILE PHYTOHORMONE TEST OBJECT

GEORGE S. AVERY, JR., AND K. LINDERSTRØM-LANG

(WITH NINE FIGURES)

### Introduction

In a previous investigation on the distribution of enzymes in plant tissue, peptidase activity was determined throughout the length of the *Hordeum* coleoptile and its inclosed first foliage leaf. The ratio between peptidase and a quantity which presumably was proportional to the protein content in the tissue was found to be fairly constant throughout the entire length of the coleoptile plus the inclosed first foliage leaf (5).

The object of the present study was to determine the distribution of peptidase throughout the *Avena* coleoptile (without its inclosed first foliage leaf) at different growth stages, both in relation to "reduced weight" of the tissue and in relation to number of cells. This particular material was chosen since the cellular morphogenesis of *Avena* is now well understood. Moreover, the coleoptile presents many interesting features in relation both to growth problems and to specific plant hormone problems.

In the germinating seedling of *Avena* it has been shown (2) that cell division in the embryonic coleoptile starts within 24 hours after the seeds are placed under suitable conditions for germination. Cell divisions are more numerous during early germination but continue with appreciable though diminishing intensity until the coleoptile is approximately 10 mm. in length (1). As long as cell division persists it goes on with equal intensity throughout the coleoptile. The coleoptile grows in length only, not in diameter (that is, growth is polarized). After a length of 10 mm. has been reached, further elongation is due almost entirely to extension of existing cells. Toward the end of the period of growth by cell extension (near maturity), the zone immediately proximal to the coleoptile tip undergoes proportionately greater extension than other portions of the coleoptile.

Thus the coleoptile is an organ in which the major period of cell division is limited to its early development and the major period of cell elongation is limited to its later development. Cells in the distal 0.5 mm. neither divide nor elongate as the coleoptile grows. They are essentially isodiametric, with large nuclei, dense cytoplasm, and small vacuoles (6). Proximal to this terminal zone, cells elongate considerably during growth of the coleoptile, and become highly vacuolate. It is this difference between the terminal cells and those proximal to them which pro-

vides the most obvious cytological basis for a physiological gradient from tip to base of the coleoptile.

The presence of an auxin gradient in the coleoptile, with a high concentration at the tip and a gradually decreasing concentration toward the base (8, 7), cannot be correlated with the synthesis of new protoplasm because there is no distinct embryonic growth center in the coleoptile at any time in its development. Hence such a gradient might be attributed to: (a) the presence of hormone-secreting cells at the tip or (b) the movement from the cotyledon or endosperm of the auxin or its "precursor" through the vascular bundles (3) to the tip, from which point it could be dispersed into the nonvascular tissues. In any case, the question which immediately arises is: to what extent does this auxin (perhaps more than one "kind") influence the metabolism of the tissues of the coleoptile?

### Investigation (methods)

PREPARATION OF MATERIAL AND GENERAL METHOD.—Seeds of *Avena sativa* L. var. Victory (Svalöf) were carefully selected and weighed for uniformity; only those between 31 and 33 mm. were used. They were soaked 1-3 hours at laboratory temperature, then placed in preparation dishes on thoroughly moistened filter paper. The dishes were placed in a dark chamber, at 25° C., and supported at a 60° angle so that the coleoptiles could grow upright without bending or coming in contact with the glass walls of the dish (fig. 1). Enough seeds were started each day so that at all times there were seedlings with coleoptiles 1.5, 4, 10, 17, and 36 mm. in length. These lengths were selected because of our knowledge of cell behavior at these lengths in the same variety (1).

When the coleoptile of a seedling had reached the desired length it was removed from the dark chamber, carefully cut away from the seedling (below the level of its attachment to the embryonic axis), and imbedded in 45° paraffin, after which it was promptly placed and kept in ice water until sectioned at 125  $\mu$  or 250  $\mu$  on a rotary microtome. Special adjustment of the microtome was necessary to cut uniformly at these thicknesses. Never more than 1 hour elapsed between the time of imbedding and completion of the cutting. Care was taken to prevent the segments from coming into contact with anything. To do this the paraffin ribbon with the coleoptile segments in it was transferred every three to five cuts to a moist chamber, where it was placed on a glass slide. One edge of the paraffin ribbon was all that touched the slide; the portion containing the segments projected over the edge. Upon completion of cutting, the coleoptile segments with surrounding paraffin were removed one by one from the moist chamber to the edge of a slide on the stage of a binocular microscope, the paraffin of the ribbon again acting as a support to keep the segment from touching anything. First the embryonic foliage leaf was removed from the central cavity, then each segment was quickly



lifted from the paraffin with the aid of a small hooked scalpel and placed in a standard volume of 30 per cent glycerin in a 250-cmm. vessel. The total length of time elapsing between sectioning the coleoptile and placing the segments in glycerin was 20-40 minutes, except when unusually large numbers of segments were

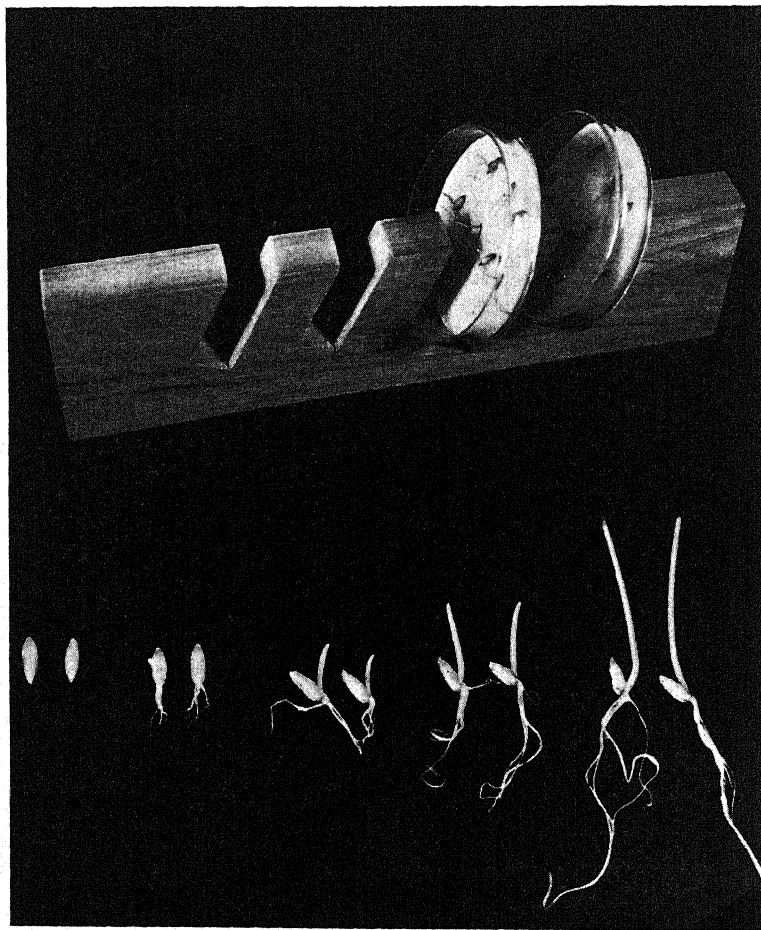


FIG. 1.—Above: Method of culturing *Avena* seedlings. Petri dishes held in nearly upright position for straight growth. Below: Seedlings in various stages of development (grown in darkness at 25° C.): 1.5 mm., 1 day old; 4 mm., 2 days old; 10 mm., 3 days; 17 mm., 4 days; 36 mm., 5 days old.

cut from the longer coleoptiles. Tests in which an additional 30 minutes to 1 hour elapsed showed no discernible differences. Peptidase determinations were made according to the method of LINDERSTRØM-LANG and HOLTER (4). Immediately upon completion of the placing of segments in glycerin, the substrate alanyl-gly-

cine (0.2M) was added with a semi-automatic pipette; and after stirring electromagnetically, the vessels were placed in an incubator at 40° C. for 4 hours, for splitting of the dipeptide to occur. (Preliminary tests showed this length of time to give good splitting with the amount of tissue involved.) At the end of the 4-hour

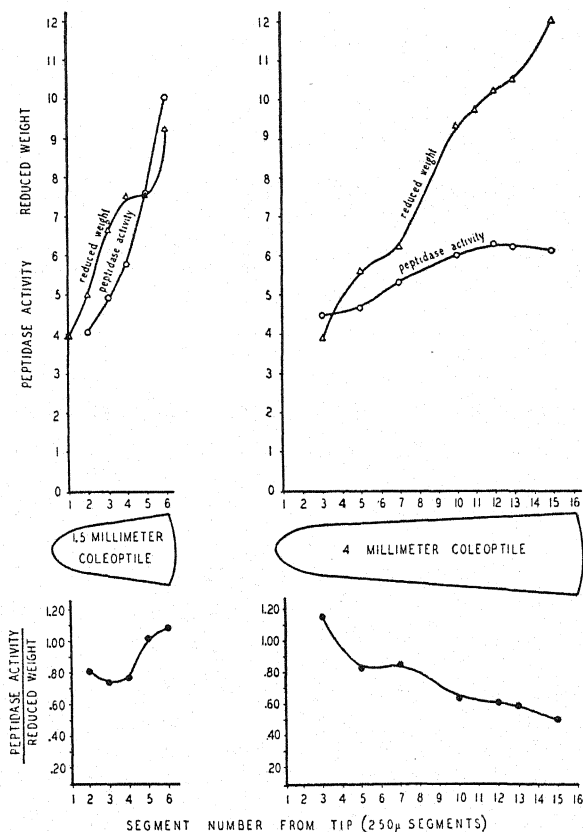


FIG. 2.—Upper left: Reduced weight and peptidase activity of successive 250- $\mu$  segments from tip to base of 1.5-mm. coleoptile, also (lower left) peptidase activity per unit weight in various portions of the coleoptile, calculated from preceding. Upper and lower right: Same, but for 4-mm. coleoptile (portion of tip extending beyond ordinate in this and other graphs was in all instances less than a full section of 250- $\mu$  thickness, hence not tested).

period, splitting was stopped by the addition of 0.05N hydrochloric acid in 95 per cent alcohol, added with an automatic pipette. To this was added approximately 150 cmm. of an acetone solution of naphthyl red, which was used as an indicator. Titration was completed by adding known amounts of hydrochloric acid in alcohol with a microburette. Cleavage of the substrate was determined in cmm. of hydrochloric acid necessary to complete the titration. Numerous segments were used for

controls. The results are expressed in arbitrary peptidase units: the increase in number of amino groups expressed as cmm. 0.05N hydrochloric acid.

THICKNESS OF SEGMENTS.—In order to make certain that under the experimental conditions there was sufficient contact between enzyme and substrate, a

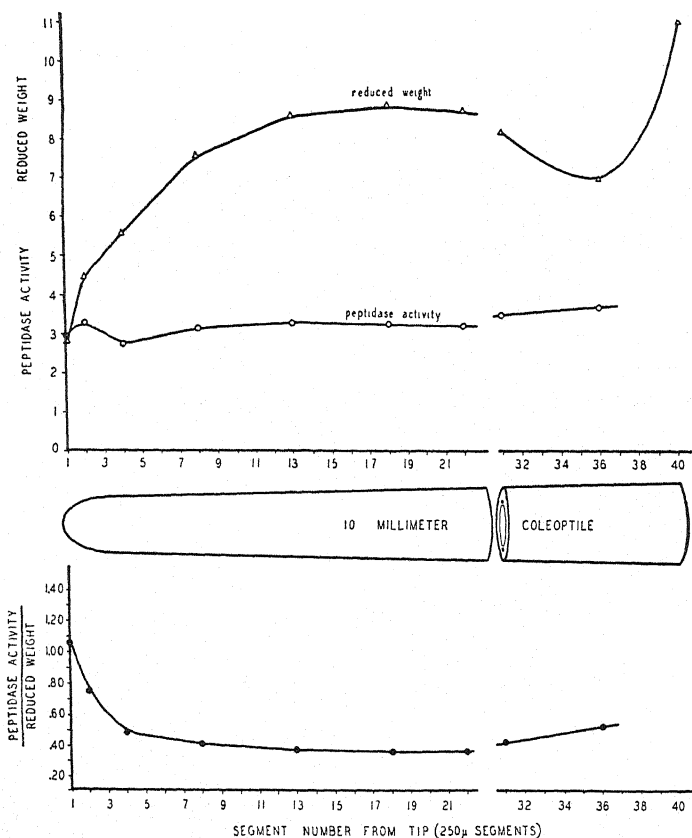


FIG. 3.—Above: Reduced weight of 250- $\mu$  segments at various levels between tip and base of 10-mm. coleoptile, also peptidase activity of corresponding segments from another 10-mm. coleoptile. Below: Peptidase activity per unit weight in various portions of coleoptile, calculated from preceding.

3.7-mm. coleoptile was cut into serial segments as follows: one segment 250  $\mu$  in thickness; two segments 125  $\mu$  in thickness; one segment 250  $\mu$ ; two segments 125  $\mu$ ; etc. The two 125- $\mu$  segments were placed in a single vessel so that alternate vessels contained a single 250- $\mu$  segment and two 125- $\mu$  segments. The results, when plotted, gave a smooth curve throughout the length of the coleoptile, indicating that both 125- $\mu$  and 250- $\mu$  segments completely liberated the enzyme.

WEIGHING OF SEGMENTS.—The imbedding and cutting procedures were used as

given, and segments from various portions of the coleoptile were placed in the gradient tube for determining "reduced weight" (figs. 2-7), according to an unpublished method of LINDERSTRØM-LANG. The so-called reduced weight represents

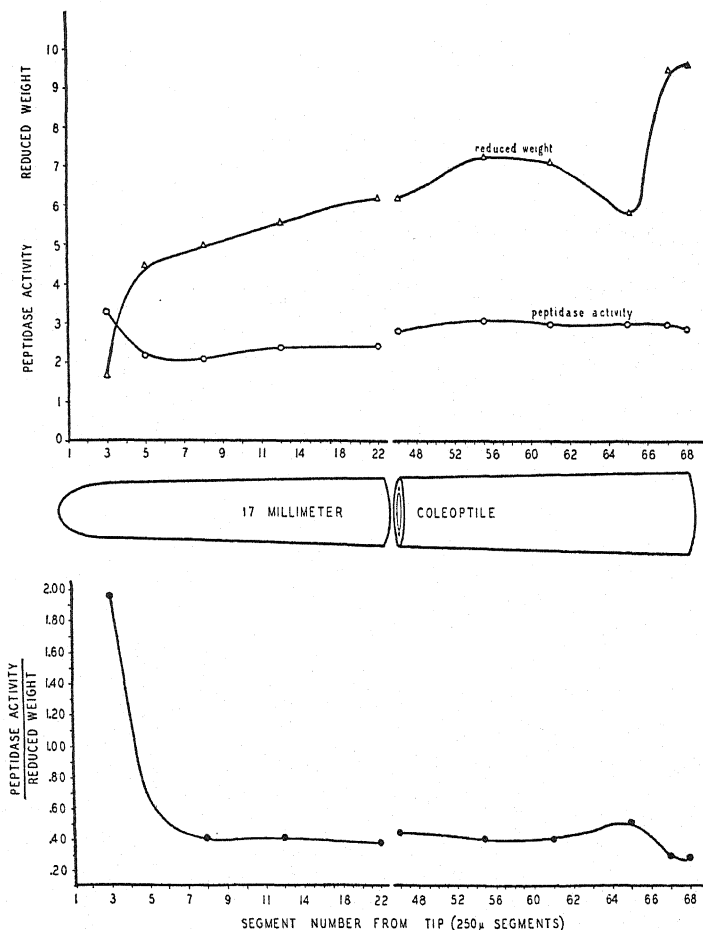


FIG. 4.—Continuation of fig. 3, but for 17-mm. coleoptile (changing scales on abscissae are of no significance; all segments for given coleoptile are of same thickness).

the solid matter of the cells; the weight of the water in the tissue therefore is not included.

**DETERMINATION OF CELL NUMBER.**—A number of representative coleoptiles were selected at the 4- and 10-mm. stages of growth, fixed in Navashin's fluid, and following the usual steps of dehydration and paraffin infiltration were imbedded and cut in serial transections at  $25\ \mu$ ; other serial longisections were cut at  $20\ \mu$ .

Cell counts were made of the total number of cells appearing in each successive  $25\text{-}\mu$  transection, and the average for each five sections was then determined by the following procedure: (a) from median longisections the average cell length in microns was determined for cells in the distal  $125\text{ }\mu$ , then for the second  $125\text{-}\mu$  portion, the third, etc., until the average cell length for each  $125\text{-}\mu$  segment of the entire coleoptile was known; (b) cell counts were then made for each  $25\text{-}\mu$  transection in the terminal five sections, totaling  $125\text{ }\mu$ , and these were averaged.<sup>1</sup> The average transection cell count was then multiplied by  $125$  to give the total length

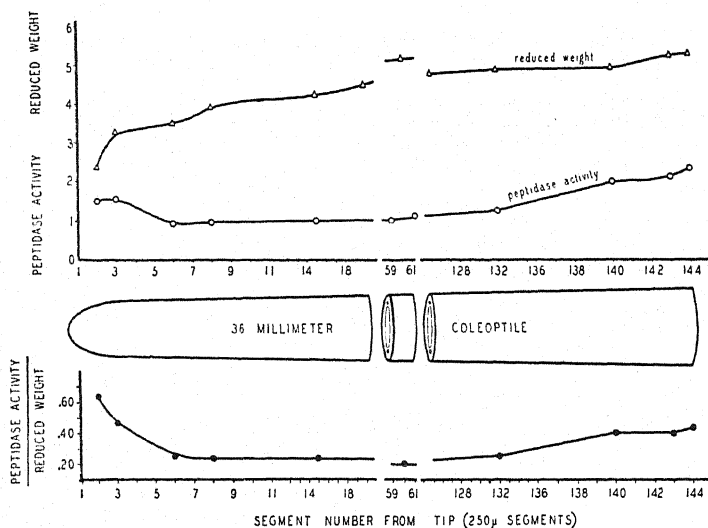


FIG. 5.—Continuation of previous figures, but for 36-mm. coleoptile

(in microns) of all cells in that particular segment, and this sum was divided by the average length of cells in the segment to give the total number of cells in that particular segment. The results of such determinations for single coleoptiles at the 4- and 10-mm. stages of development appear in figures 8 and 9.<sup>2</sup>

### Investigation (results)

**1.5-MILLIMETER COLEOPTILES.**—Coleoptiles 1.4–1.6 mm. were prepared as indicated, cut into  $250\text{-}\mu$  segments, and peptidase determinations made on successive sections. There were slight differences in the magnitude of enzyme activity

<sup>1</sup> Vascular bundles not included; in a 4-mm. coleoptile, at the proximal end of the tip  $125\text{-}\mu$  segment, the bundles constitute 0 per cent of the area of a transection of the coleoptile; at the base of the third segment, 3.4 per cent of the area; fifth segment, 5 per cent of the area; tenth segment, 8.7 per cent; and fifteenth segment, 4.8 per cent.

<sup>2</sup> A statistical study of cell number in the coleoptile is now in progress.

in different coleoptiles, but in all instances the distribution of the enzyme agreed with data given for the 1.5-mm. coleoptile (fig. 2, upper left). With the exception of the tip-most segment, peptidase activity in 1.5-mm. coleoptiles was higher throughout than in any of the older coleoptiles.

On the basis of the reduced weights determined for a 1.6-mm. coleoptile, the peptidase activity per unit weight decreases from base to tip (fig. 2, lower left). At this stage of development, cells throughout the coleoptile are undergoing more rapid cell division, and therefore more rapid synthesis of protoplasm, than at any later stage in their growth.

**4-MILLIMETER COLEOPTILES.**—At this stage of coleoptile growth peptidase activity was still high throughout (fig. 2, upper right), but except for the tip-most section the activity is not so great as in the 1.5-mm. coleoptiles. Reduced weights of successive segments from tip to base of a 4.1-mm. coleoptile are given in figure 2 also, and on the basis of these determinations the peptidase activity per unit weight (fig. 2, lower right) is highest at the tip and decreases steadily toward the base. This is the reverse of the gradient noted in coleoptiles 1.5 mm. in length.

**10-, 17-, and 36-MILLIMETER COLEOPTILES.**—Peptidase activity and reduced weights of selected segments from coleoptiles of these lengths are given in figures 3-5. In each instance the gaps in the abscissa indicate that for these segments enzyme activity was not determined. Reduced weights were obtained at equal distances throughout the greater part of the length of the coleoptile, and even though omitted from the graphs at the gaps indicated, the general trend may be observed. The higher reduced weights of segments at or near the base of the 10- and 17-mm. coleoptiles agree with the determinations for younger coleoptiles, but

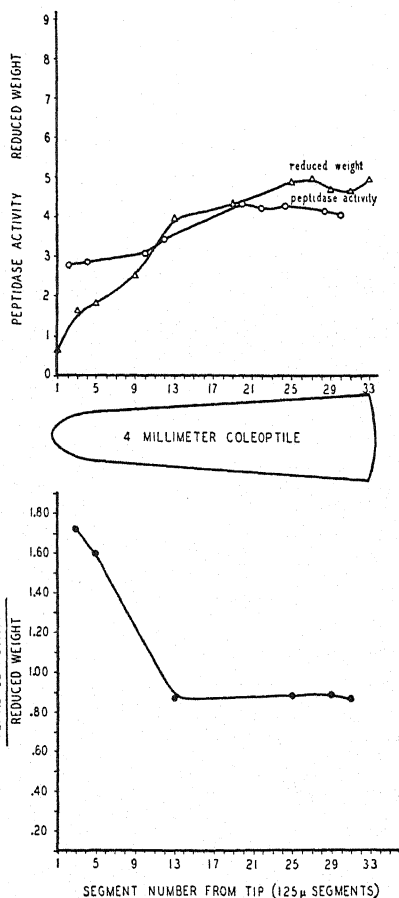


FIG. 6.—Above: Coleoptile 4 mm. in length, cut into 125-μ segments. Alternate segments used for determining reduced weights, and peptidase activity. Below: Peptidase activity per unit weight in various portions of coleoptile, calculated from preceding.

just above the basal segments weight decreases somewhat, only to rise again through the middle portion of the organ. Reduced weights of selected segments from the 36-mm. coleoptile show a very gradual rise from tip toward base, with but slightly increased weight through the middle of the coleoptile.

In general, the reduced weight of segments throughout the length of the coleoptile decreases with increasing age. Peptidase activity also decreases as the age of the coleoptile increases, but is always greater at the tip and base of the

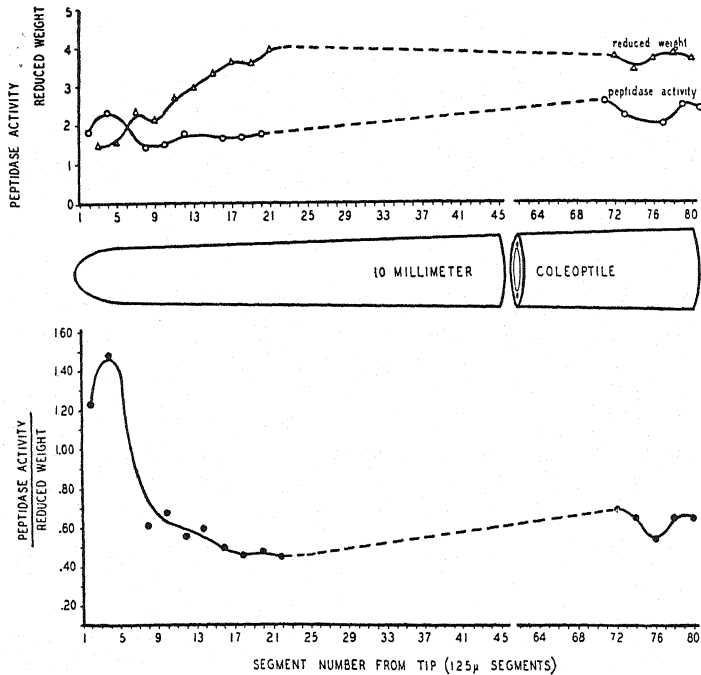


FIG. 7.—Continuation of fig. 6, but for 10-mm. coleoptile

coleoptile than through the middle portion. Peptidase activity per unit weight is much greater in segments near the tip, particularly in the 10- and 17-mm. coleoptiles.

4- AND 10-MILLIMETER COLEOPTILES, ALTERNATE SEGMENTS WEIGHED AND PEPTIDASE ACTIVITY DETERMINED.—In all the foregoing experiments, reduced weights and peptidase activity were determined on different coleoptiles, but of the same size for each age group. In this experiment a given coleoptile was cut into 125- $\mu$  segments. Alternate segments were used for determining reduced weights, the remaining ones for determining peptidase activity. Hence peptidase activity per unit weight was obtained for segments throughout the length of a

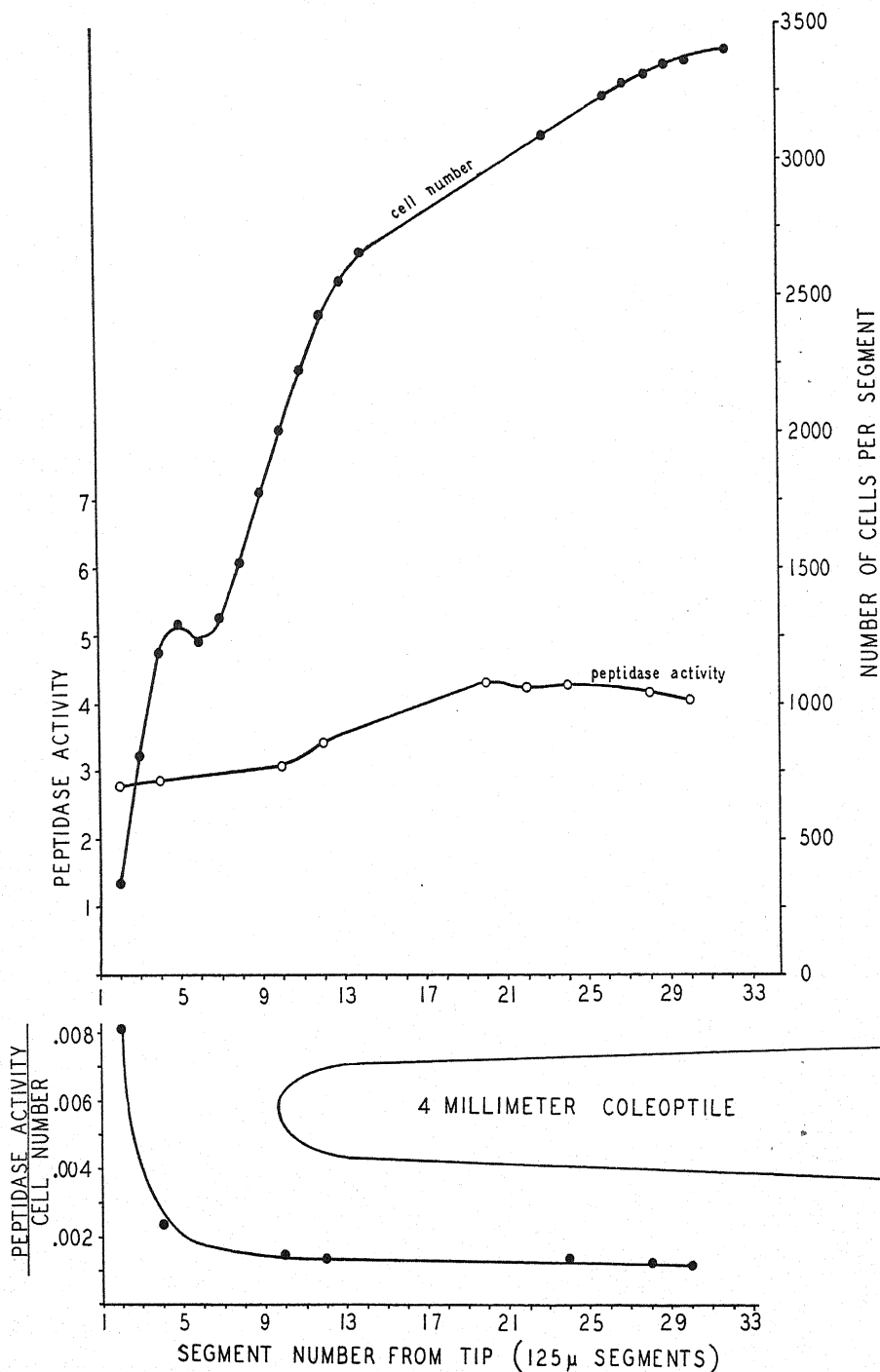


FIG. 8.—Above: Peptidase activity same as in fig. 6, but including number of cells per 125- $\mu$  segment (vascular cells omitted, but these occupy only ca. 5%, on average, of cross-sectional area). Below: Peptidase activity per cell in various portions of coleoptile, calculated from preceding.



given coleoptile (figs. 6, 7). The results corroborate those reported, and bring out even more sharply the fact that peptidase activity per unit weight of tissue is much greater at the tip of the coleoptile.

PEPTIDASE ACTIVITY IN RELATION TO NUMBER OF CELLS PER SEGMENT.—Figure 8 (above) shows that the number of cells per 125- $\mu$  segment increases considerably—

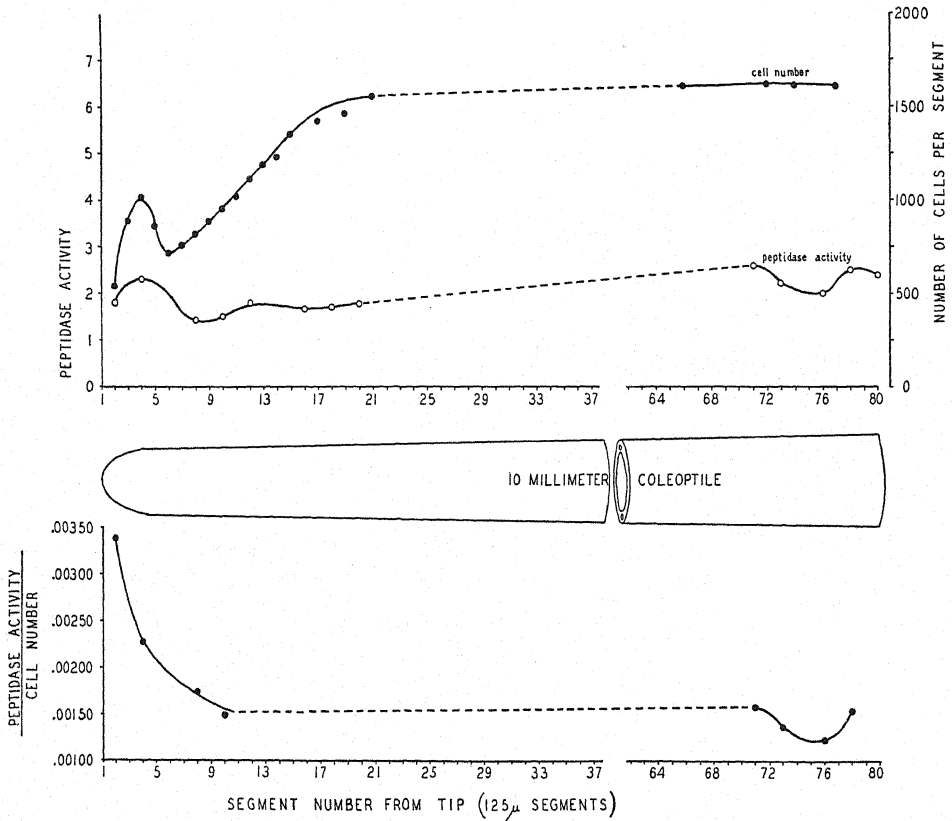


FIG. 9.—Continuation of fig. 8, but for 10-mm. coleoptile

more than tenfold—from tip to base in a 4-mm. coleoptile. Peptidase activity, on the other hand, is only slightly greater in segments from the middle and basal portions of a 4-mm. coleoptile. On a per cell basis, this means that the peptidase activity is considerably higher at the tip than in other portions of the coleoptile (fig. 8, below).

Cell number per segment for a 10-mm. coleoptile is given in figure 9 (above). While the total number of cells in the whole coleoptile is much greater than in one

4 mm. in length, there are fewer cells per segment because the average cell is approximately twice as long. Peptidase activity per cell is here again much higher at the tip than in other portions (fig. 9, below).

The "pore" of the coleoptile occurs in the fourth segment; around it the cells are somewhat smaller. Distal to the pore there is little or no cell elongation as the coleoptile grows, but cells proximal to it stretch appreciably. This accounts for the jog in the curve (fig. 9, above).

AUXIN IN COLEOPTILES.—Tips were removed from coleoptiles of the same age groups as the preceding and their auxin content tested by immediately placing them unilaterally on decapitated test coleoptiles for 2 hours, according to the method of WENT. Each figure represents the average of forty-eight coleoptile tips, from coleoptiles of the lengths indicated.

COLEOPTILE LENGTH

	1.5 MM	3 MM	4 MM	10 MM	17 MM	36 MM
Auxin concentration (in degrees <i>Avena</i> curvature) . . . . .	2.1	4.8	6.2	9.9	9.9	8.9

It is clear that there is an increase in auxin concentration in tips of coleoptiles between 1.5 and 10 mm. in length; there is no further increase in the 10–17-mm. stages; and there is a slight decrease as the coleoptile nears maturity (36 mm.).

As for the distribution of auxin in coleoptiles, THIMANN (8) (see also SÖDING, 7), working with coleoptiles 25 mm. in length, showed the presence of an auxin gradient from tip to base; highest concentrations were present at the tip, with decreasing amounts at successively lower levels. The higher concentration of auxin in the tips is correlated with higher peptidase activity per unit weight at the tip; also higher peptidase activity per cell.

### Discussion

That the distal ends of coleoptiles 1.5 mm. in length were found low in auxin, by the method used, cannot be shown at this time to have definite bearing on the fact that peptidase is relatively low in the tips of such coleoptiles. Nor at this time can the higher auxin content of the tips of 4-mm. and older coleoptiles be causally related to the higher peptidase activity per unit weight of tissue, or per cell. These points take on added interest, however, in the light of the approximate parallelism of the peptidase and auxin gradients in older coleoptiles. Whether auxin in higher concentrations can effectively act as an enzyme activator remains to be demonstrated, but suggestive evidence is at hand.

Physiologically active substances other than auxin also have been demonstrated

to be present in higher concentrations in the tip than elsewhere in the coleoptile, for example, ascorbic acid and vitamin B<sub>1</sub>.

The most important fact on which to base an explanation of the high peptidase activity of the coleoptile tip is the character of the cells in the terminal 0.5 mm. They differ sharply from cells proximal to them; as the coleoptile grows they undergo no division and very little enlargement. As a result they are richer in protoplasm, that is, they have large nuclei and dense cytoplasm. Because of this obvious protoplasmic difference we feel that an investigation of the ratio of peptidase activity to nitrogen content in the tip cells is called for, and such work will soon be under way. The relation between such histological differences, which undoubtedly find their chemical images in peptidase distribution and production or transport of auxin in the coleoptile tip, is more obscure.

### Summary

1. *Avena* seedlings were grown in darkness at 25° C. on moist filter paper in preparation dishes. When the coleoptiles were 1.5, 4, 10, 17, and 36 mm. in length, they were uniformly sectioned at 125 or 250  $\mu$  on a rotary microtome. Peptidase determinations were made on these segments, and their reduced weights were obtained by use of the gradient tube. Cell counts were made for successive 125- $\mu$  segments of coleoptiles 4 and 10 mm. in length. On a per segment basis, peptidase activity, reduced weight, and cell number were found to decrease in progressively older coleoptiles.

2. For any given coleoptile 4 mm. or more in length, enzyme activity per unit weight of tissue, or per cell, was consistently greater at the tip. Correlations between morphological structure and auxin and peptidase gradients in the coleoptile are pointed out.

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# AGGLUTINATION TEST APPLIED TO STRAINS OF PHYTOMONAS STEWARTI

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## Introduction

Strains of the sweet-corn wilt bacterium, *Phytomonas stewarti* (E.F.S.) Bergey *et al.*, that differ in virulence, colony characteristics, color, and physiological abilities have been described in recent years (8, 13, 14, 15, 24, 27). The bacteria from wilted plants in different sections of the United States have been classified into three main types by IVANOFF *et al.* (7), but no attempt has been made to determine the relationship of these and other strains. Studies were undertaken, therefore, to determine whether some of these strains differed in their serological properties, and if so, what their relationships to one another might be. This paper reports the results of cross agglutination tests made with strains from different geographical areas and with variant types obtained from some of the strains. Data are also presented on the agglutination of ninety field isolates of *P. stewarti* and of several other species of wilt bacteria.

## Material and methods

Fifteen strains of *P. stewarti* were used as antigens for producing immune sera. These strains were selected because they represented different geographical areas as well as different cultural and pathogenic types. Their origin and distinctive characteristics were as follows:

B-11.—A highly virulent strain isolated at Princeton, New Jersey, in September, 1935 (14). The culture had lost some of its virulence during the 3 years it had been maintained on nutrient-dextrose agar.

B-1011a.—An almost avirulent single-colony isolate obtained from B-1011 (14), which was a highly virulent strain obtained from B-11.

B-1311.—A virulent single-colony isolate obtained from B-1011. At one time (15) this strain was the most virulent of all progeny obtained from strain B-11, but it had lost some of its virulence at the time of these experiments.

B-1611.—An avirulent single-colony isolate obtained from B-11 in July, 1936.

B-1111.—An almost avirulent single-colony isolate obtained from B-11. This culture represents a type that is weakly virulent, apparently because of its inability to use inorganic nitrogen (14).

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- B-102.—A highly virulent strain isolated from a naturally infected resistant inbred line of maize (6-215) at Princeton, in August, 1928.
- B-103.—A highly virulent strain isolated from an extremely susceptible inbred line (C-18) at Princeton, in August, 1938.
- B-107.—A highly virulent strain isolated from maize by Dr. ELLIOTT at Orizaba, Mexico, in December, 1938.
- B-108.—A highly virulent strain isolated by Dr. ELLIOTT from maize in Lucas County, Ohio, in October, 1937.
- B-109.—A highly virulent strain isolated by Dr. ELLIOTT from the flea beetle vector, *Chaetocnema pulicaria* Melsh., at Arlington, Virginia, in November, 1938.
- B-8.—A weakly virulent strain from New York used by Dr. WELLHAUSEN as the parent strain for his host passage studies (24). It differed from all other cultures except B-92 in producing a dry, crustaceous type of growth on agar.
- B-96.—A highly virulent isolate obtained by Dr. WELLHAUSEN (24) from B-8 after a series of passages in a resistant inbred line of maize designated as OSF.
- B-91.—A highly virulent isolate obtained from B-8 by a series of host passages similar to those described for B-96 (24).
- B-94.—An avirulent single-colony isolate obtained from B-96 after it had been cultivated on nutrient-dextrose agar slants for 18 months.
- B-92.—A weakly virulent isolate obtained by Dr. WELLHAUSEN (24) from culture B-8 after a series of passages in a susceptible inbred line of maize designated as GB.

These different strains were purified by single-colony isolation at the beginning of the experiments in order to eliminate any variant types that might have developed in culture. The isolates were stored on nutrient-dextrose agar at 8° C., and subcultures from this stock were used in all subsequent tests. The cultures were tested for virulence as described elsewhere (16) immediately after purification and again at the conclusion of the experiments. No decided changes were observed, so only data on the latter test are reported (table 1).

Immune sera for these strains were obtained from rabbits weighing between 2 and 3.6 kilos. All rabbits were bled at the beginning of the experiment and found to be free of agglutinins for the different bacterial strains. Bacteria grown on potato-dextrose agar slants and suspended in sterile saline solution (0.85 per cent NaCl) were injected intraperitoneally at 4-day intervals. After injections of 1, 1, 3, and 4 cc. of suspension, an injection was made intravenously but the rabbits suffered severe shock and four of the fifteen died. The sixth and final injection

was made intraperitoneally and the rabbits were bled from an ear vein 12 days later. The rabbits lost 0.2-0.7 kilos in weight during the period of injection; some continued to lose until the time of bleeding, and general recovery in weight did not occur until 12 days later. The blood was placed in an icebox overnight, the serum removed by pipette and frozen until it was used for tests.

The serum was diluted 1:20, 1:40, 1:80, 1:160, 1:320, 1:640, 1:1280, 1:2560, and 1:5120 with sterile saline and inoculated with sufficient bacteria to give an opalescent suspension. The bacteria were grown for 24 hours on nutrient-dextrose

TABLE 1

VIRULENCE OF *P. STEWARTI* STRAINS FOR 7-DAY-OLD SWEET-CORN SEEDLINGS

STRAIN TESTED	PERCENTAGE OF LEAVES INVADED IN DIFFERENT TESTS						DRY WEIGHT PER PLANT (GM.)
	1	2	3	4	5	AVERAGE	
B-II.....	75.2	67.8	77.4	75.5	77.1	74.6 ± 3.9	0.31 ± 0.05
B-1011a.....	0.0	0.0	0.0	0.0	0.0	0.0 ± 0.0	0.46 ± 0.03
B-1311.....	70.1	67.3	59.8	58.0	54.9	62.0 ± 6.4	0.33 ± 0.05
B-1611.....	0.0	0.0	0.0	0.0	0.0	0.0 ± 0.0	0.46 ± 0.03
B-1111.....	0.0	2.6	0.0	1.8	0.0	0.9 ± 1.2	0.43 ± 0.05
B-102.....	90.4	66.7	79.8	76.9	79.4	78.6 ± 8.5	0.32 ± 0.05
B-103.....	67.9	77.4	83.0	69.0	76.4	74.7 ± 6.3	0.33 ± 0.05
B-107.....	87.4	85.3	95.1	89.9	87.5	89.0 ± 3.8	0.28 ± 0.05
B-108.....	84.3	90.5	93.1	82.9	92.0	88.6 ± 4.6	0.22 ± 0.03
B-109.....	60.4	76.3	67.3	73.1	69.0	69.2 ± 6.0	0.32 ± 0.05
B-8.....	34.0	34.8	32.5	36.2	28.2	33.1 ± 3.1	0.40 ± 0.01
B-96.....	56.6	82.2	65.4	54.9	77.9	67.4 ± 12.4	0.32 ± 0.05
B-91.....	76.5	76.9	71.6	88.9	80.0	78.8 ± 6.4	0.30 ± 0.09
B-94.....	0.0	0.0	0.0	0.0	0.0	0.0 ± 0.0	0.49 ± 0.09
B-92.....	36.2	33.1	28.0	42.3	33.9	34.7 ± 5.2	0.44 ± 0.06
Uninoculated controls.....	0.0	0.0	0.0	0.0	0.0	0.0 ± 0.0	0.51 ± 0.06

agar, suspended in saline and allowed to stand overnight at 8° C., so that all particles of agar and bacterial clumps might settle out. They were then thoroughly mixed with the serum, incubated at 40° C. for 2 hours, and stored overnight at 8° C. before final observations on agglutination were made. The degree of agglutination was recorded as + + + + for complete clearing, + + + for pronounced agglutination without complete clearing, + + for definite agglutination, + for slight agglutination, ± for doubtful agglutination, and - for no agglutination. The doubtful agglutinations consisted of clouding of the serum without the appearance of macroscopically visible clumps. Clumps of 3-30 cells could be detected by microscopic examination, but only macroscopic tests were made as a general rule.

## Experimental results

### AGGLUTINATION OF FIELD ISOLATES IN IMMUNE SERUM

In order to determine whether the strains prevalent in maize fields at Princeton, New Jersey, differed in their serological characteristics, ninety single-colony isolates were tested for agglutination in the immune sera of the highly virulent B-96 and slightly virulent B-1111. The field isolates were obtained by methods described elsewhere (16) from leaf lesions on naturally infected plants of the various inbred lines listed in table 2. These inbred lines ranged in susceptibility from the completely susceptible C-18 to the highly resistant OSF in the order in which they are listed. Isolates were usually obtained from two or more plants of each inbred line, and two to six isolates from each plant were tested. All the isolates belonged, in so far as could be determined, to the B type described by IVANOFF *et al.* (7). All were highly virulent immediately after isolation, but at the time of the tests, 4 months later, some had partially lost virulence for Golden Bantam seedlings.

The ninety isolates differed widely in their ability to agglutinate in the immune sera. In order to save space, data on only thirty of these isolates are presented. The data in table 2 serve to illustrate the general differences observed. Many of the isolates, such as 3, 4, 6, 15, 25, 26, 28, and 30, agglutinated well in both sera even at dilutions of 1:1280 or more; it is obvious that both sera were fairly potent. It should be pointed out, however, that the B-96 serum caused more complete agglutination at the 1:20 and 1:40 dilutions than did the B-1111 serum. Some of the isolates (2, 11, 19, 21, 22, 23, 29) agglutinated better in B-96 than in B-1111 serum. On the other hand, isolates such as 1, 7, 8, 12, 16, and 27 agglutinated readily in diluted B-1111 serum but poorly in B-96 serum. Isolates such as 9, 10, 13, 17, 18, and 24 agglutinated poorly in both sera, and isolates 14 and 20 failed to agglutinate in either.

The bacteria from resistant and susceptible inbred lines gave similar agglutination reactions, and no consistent difference could be observed in the tendency for atypical strains to occur in the two types of hosts. As a general rule, isolates from the same plant had similar serological characteristics, but in some instances isolates such as 14 and 15 from the same lesion were entirely different. Frequently the isolates from different plants of the same inbred line were just as different from one another as they were from isolates obtained from some other line.

The data on these field isolates show that the agglutination test cannot be used unconditionally as a means of identifying *P. stewartii* cultures. Obviously more than one serum should be tested, but even then the failure of a strain to agglutinate would not disprove its identity. Further tests on morphology, physiology, and virulence would have to be resorted to in identifying these non-agglutinating strains.



TABLE 2

## AGGLUTINATION OF BACTERIA ISOLATED FROM NATURALLY INFECTED INBRED LINES OF MAIZE AT PRINCETON, IN 1938

ISOLATE TESTED			AGGLUTINATION OF BACTERIA IN IMMUNE SERUM																	
EXPERI- MENT NO.	FROM INBRED LINE	VIRULENCE		B-96 SERUM DILUTED*								B-1111 SERUM DILUTED								
		PERCENTAGE LEAVES	KILLED	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	
1.....	C-18a†	87.7	51.2	++	±	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+
2.....	C-18a†	20.0	1.1	++	++	++	++	++	++	++	++	++	++	++	+	+	+	+	+	+
3.....	C-18b	96.1	55.8	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
4.....	C-18b	87.5	37.5	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
5.....	6-595a	84.4	53.3	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
6.....	6-565b	91.4	64.3	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
7.....	GBa	96.2	62.8	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
8.....	7-40a	80.7	49.4	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
9.....	7-40b	98.4	77.8	+	±	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+
10.....	7-40b	98.5	82.4	+	±	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+
11.....	7-306a	90.7	53.3	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
12.....	6-573a	92.3	65.4	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
13.....	6-573b	93.0	60.6	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
14.....	6-214a	85.3	56.0	±	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+
15.....	6-214a	66.7	32.3	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
16.....	6-214b	83.3	35.7	++	±	-	-	-	-	-	-	-	-	-	++	++	++	++	++	++
17.....	6-195a	89.2	60.2	++	-	-	-	-	-	-	-	-	-	-	++	++	++	++	++	++
18.....	6-195a	95.3	76.5	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
19.....	6-195b	77.4	58.3	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
20.....	Y-30a	82.7	38.7	-	-	-	-	-	-	-	-	-	-	-	±	±	±	±	±	±
21.....	6-1494a	76.6	41.6	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
22.....	6-1494b	87.7	56.8	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
23.....	6-1494b	72.6	43.2	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
24.....	6-215a	93.8	63.8	+	++	++	++	++	++	++	++	++	++	++	±	±	±	±	±	±
25.....	6-215b	85.1	44.8	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
26.....	6-215b	69.8	30.2	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
27.....	7-1992a	88.9	52.8	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
28.....	7-1992b	86.1	56.9	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
29.....	7-1992b	77.9	42.9	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
30.....	OSFa	92.3	62.8	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++

\* Data on 1:5120 dilution and saline controls omitted, since all tests were negative.

† Small letters indicate different plants of each inbred line; for example, this is plant "a" of inbred line C-18.

RECIPROCAL AGGLUTINATION TESTS ON FIFTEEN STRAINS DIFFERING  
IN VIRULENCE AND CULTURAL CHARACTERISTICS

The discovery that serologically distinct variants could be isolated from the same leaf lesion raised the question of whether the plant had been infected originally by a single strain which later produced serological variants or was infected originally by more than one strain. Such a question cannot be answered until it is known whether all the progeny of a given serological type have the same reaction in immune serum. The cross agglutinations of several variant strains obtained from cultures B-11 and B-8 were tested in order to gain information on this point.

TABLE 3  
MAXIMUM DILUTION OF ELEVEN IMMUNE SERA IN WHICH  
FIFTEEN STRAINS OF *P. STEWARTI* AGGLUTINATED\*

STRAIN TESTED	MAXIMUM DILUTION AT WHICH AGGLUTINATION OCCURRED IN ANTISERUM OF STRAIN										
	B-11	B-1011a	B-1611	B-1111	B-102	B-107	B-108	B-8	B-96	B-91	B-94
B-11.....	1:160	1:40	—†	—	—	—	1:40	—	1:40	1:40	1:40
B-1011a.....	1:640	1:320	(1:40)‡	1:40	1:320	1:80	1:80	1:40	1:320	1:1280	1:640
B-1311.....	1:160	1:40	—	—	—	—	1:40	—	1:80	1:40	1:40
B-1611.....	1:160	1:640	1:1280	1:2560	1:320	1:640	1:320	1:2560	1:160	1:640	1:640
B-1111.....	1:1280	1:640	1:320	1:2560	1:1280	1:320	1:640	1:2560	1:640	1:1280	1:2560
B-102.....	1:2560	1:2560	1:1280	1:2560	1:5120	1:2560	1:1280	1:2560	1:5120	1:2560	1:5120
B-103.....	1:2560	1:40	1:20	1:80	1:120	(1:80)	1:80	1:320	1:40	1:40	1:160
B-107.....	1:5120	1:160	1:160	1:640	1:320	1:320	1:640	1:320	1:160	1:160	1:2560
B-108.....	1:320	1:40	1:40	1:40	1:80	1:160	1:80	1:80	1:80	1:320	1:1280
B-109.....	1:160	1:40	1:80	1:80	1:20	(1:20)	1:320	1:80	1:160	1:40	1:160
B-8.....	1:2560	1:1280	1:1280	1:2560	1:2560	1:2560	1:1280	1:5120	1:2560	1:2560	1:1280
B-96.....	1:320	1:320	(1:320)	(1:40)	(1:80)	(1:80)	1:160	(1:160)	1:160	1:320	1:80
B-91.....	1:160	1:80	(1:80)	—	(1:40)	(1:80)	1:40	—	1:40	1:80	1:40
B-94.....	1:160	1:160	(1:80)	—	—	—	1:40	—	1:40	1:40	1:40
B-92.....	1:1280	1:640	1:1280	1:1280	1:640	1:1280	1:320	1:1280	1:160	1:640	1:160

\* None of the strains agglutinated when incubated in saline solution.

† No agglutination occurred in either test or at most a doubtful reaction at 1:20 dilution.

‡ Dilution in parentheses represents very faint reaction at this dilution in one test and negative reaction in the other.

Their reactions were also compared with those of several other strains obtained from different geographical areas. The original plan was to compare all fifteen cultures listed in the section on materials, but, as already mentioned, four of the rabbits succumbed and only eleven sera were obtained.

Agglutinations of all fifteen strains in the eleven different sera were tested. Since it was not feasible to test all 165 possible combinations at once, the tests were run on two different dates. In the preliminary tests, half the cultures were tested at one time and the remainder 2 days later. On the repetition, five of the sera were tested against all cultures on the first date and the six remaining sera tested later. Since very similar results were secured in the repetitions, the data are presented without reference to time of test in tables 3, 4, and 5.

The data on the agglutination of all strains in each serum are too voluminous to be presented in a single table, but a summary of the maximum dilution at which

TABLE 4

AGGLUTINATION OF FIFTEEN STRAINS OF *P. STEWARTI* IN ANTISERUM FOR STRAIN B-II\*

STRAIN TESTED	AGGLUTINATION OF BACTERIA IN SERUM DILUTED								
	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120
B-II.....	++++ ++++	++++ ++++	++ ++++	± +	— ±	— —	— —	— —	— —
B-1011a.....	++++ ++++	++++ ++++	++ ++++	++ +	± +	— +	— +	— —	— —
B-1311.....	++++ ++++	++++ ++++	++ ++++	+	— —	— —	— —	— —	— —
B-1611.....	++++ +++	++++ +++	+ +++	± ±	— —	— —	— —	— —	— —
B-1111.....	++++ +++	++++ +++	+++ ++	+++ +	++ +	++ +	++ —	++ —	++ —
B-102.....	++++ ++++	++++ ++++	++++ ++++	++++ ++++	+++ ++	++ +	++ +	++ +	— —
B-103.....	++++ ++++	++++ ++++	++ ++++	± +	++ ±	++ ±	± ±	± ±	— +
B-107.....	++++ ++++	++++ ++++	++++ +++	++ +++	++ ++	++ +	++ +	++ +	— +
B-108.....	++++ ++++	++++ ++++	+++ ++++	++ +	— +	— +	— —	— —	— —
B-109.....	++++ ++++	++++ ++++	++++ ++++	++ +	± —	— —	— —	— —	— —
B-8.....	++ ++	+++ +++	+++ +++	+++ +++	+++ +	+++ +	+++ +	± +	++ —
B-96.....	++++ ++++	++++ ++++	++++ +++	++ +	— +	— ±	— —	— —	— —
B-91.....	++++ +++	++++ ++++	++ ++++	— +	— —	— —	— —	— —	— —
B-94.....	++++ +++	++++ ++++	++ ++++	— +	— —	— —	— —	— —	— —
B-92.....	++++ +++	++++ ++++	++ ++++	++ +	++ +	++ +	— +	— —	— —

\* None of the strains agglutinated when incubated in saline solution.

agglutination occurred in each test is presented in table 3. In the few instances in which the repetitions were not in exact agreement, a value intermediate between those resulting from the two observations was recorded. The data show that the

strains differed considerably in their agglutination in the different sera. Strains B-8, B-102, B-1111, B-1611, and B-107 agglutinated in all sera and usually at

TABLE 5

AGGLUTINATION OF FIFTEEN STRAINS OF *P. STEWARTI* IN ANTISERUM FOR STRAIN B-8\*

STRAIN TESTED	AGGLUTINATION OF BACTERIA IN SERUM DILUTED								
	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120
B-11.....	— ±	— —	— —	— —	— —	— —	— —	— —	— —
B-1011a.....	++ ±	± ±	— ±	++ —	— —	— —	— —	— —	— —
B-1311.....	— —	— —	— —	— —	— —	— —	— —	— —	— —
B-1611.....	++ +	+++ +	++ +	++ +	++ +	++ +	++ +	± +	— +
B-1111.....	++ ++	++ ++	++ +	++ +	++ +	++ +	++ +	± +	— —
B-102.....	+++ ++	+++ ++	++ +	++ +	++ +	++ +	++ +	± +	— —
B-103.....	++ +	++ +	++ ±	++ ±	++ ±	± —	— —	— —	— —
B-107.....	+++ ++	++ ++	++ +	± +	± ±	— ±	— —	— —	— —
B-108.....	++ +	++ +	± ±	— ±	— —	— —	— —	— —	— —
B-109.....	++ +	++ +	++ ±	± —	± —	— —	— —	— —	— —
B-8.....	+++ ++	+++ ++	+++ ++	+++ ++	+++ ++	++ +	++ +	++ +	++ ±
B-96.....	— ±	— +	— +	— +	— ±	— ±	— —	— —	— —
B-91.....	— ±	— —	— —	— —	— —	— —	— —	— —	— —
B-94.....	— ±	— —	— —	— —	— —	— —	— —	— —	— —
B-92.....	— +	++ +	++ +	++ ±	++ +	++ +	++ ±	++ —	— —

\* None of the strains agglutinated when incubated in saline solution.

the high titers of 1:1280 to 1:5120. Other strains, such as B-11, B-1311, B-96, B-91, and B-94, failed to agglutinate in some of the sera and agglutinated poor-

ly in others. The remaining strains agglutinated in all sera but usually only at the lower dilutions.

The data in table 3 show that closely related strains do not necessarily have the same serological properties. Cultures B-11, B-1011a, B-1311, B-1611, and B-1111 were all single-colony isolates from the same New Jersey culture, yet B-1611 and B-1111 agglutinated much more easily than the other three. Strain B-8 agglutinated much more readily in dilute immune serum than did B-96, B-91, B-94, and B-92, which had been derived from it. Strains derived from the same culture did not agglutinate better in antiserum for each other than did strains obtained from different sources. Some cultures, such as B-11 and B-108, did not agglutinate even in their own sera at high dilutions. Since the failure of these strains to agglutinate in dilute serum cannot be due to lack of specificity, it is probably attributable to their ability to resist agglutination.

The data in table 3 show that some sera were more effective than others. As a general rule the easily agglutinating strains (B-8, B-102, B-107, B-1611, and B-1111) produced serum that agglutinated similar strains but failed to agglutinate B-11, B-1311, B-96, and B-94. These poorly agglutinating strains, on the other hand, produced immune serum that agglutinated all fifteen strains.

Some of the differences observed between the two types of sera are not obvious from the general summary in table 3, so detailed data on the serum for strains B-11 and B-8 are presented in tables 4 and 5. The B-11 serum caused a heavy agglutination of all strains but lost potency for some strains when diluted more than 1:80. Strains such as B-8, B-1111, B-107, and B-102 were agglutinated at higher dilutions. The immune serum for B-8 (table 5) caused its homologous culture to agglutinate well at all dilutions up to 1:5120. The other easily agglutinating strains were similarly affected, but in no case was agglutination at the 1:20, 1:40, and 1:80 dilutions as heavy as that observed in B-11 serum. Several strains failed to agglutinate even at the 1:20 dilution of this serum.

#### AGGLUTINATION OF OTHER SPECIES OF WILT BACTERIA IN IMMUNE SERUM FOR *P. STEWARTI*

A number of wilt-producing bacteria are known to be capable of invading sweet corn and inducing mild symptoms (25, 16). Several species of these organisms were tested for agglutination in the immune sera for B-108, B-8, and B-94 to determine whether the reaction was specific for *Phytophthora stewartii*. The species used were *P. insidiosa* (McC.) Bergey *et al.*, *P. michiganensis* (E.F.S.) Bergey *et al.*, *P. solanacearum* (E.F.S.) Bergey *et al.*, *P. sepedonicum* (Spieck.) Bergey *et al.*, *P. flaccumfaciens* (Hedges) Bergey *et al.*, and the motile bacterium isolated from nitrogen-starved maize seedlings by McNEW and SPENCER (16). With the exception of *P. flaccumfaciens*, two isolates of each species were obtained from different sources and tested.

TABLE 6

AGGLUTINATION OF *P. STEWARTI* AND OTHER SPECIES OF WILT-PRODUCING BACTERIA IN ANTISERUM FOR STRAINS B-108, B-8, AND B-94\*

BACTERIA TESTED	ANTI-SERUM OF STRAIN	AGGLUTINATION OF BACTERIA IN SERUM DILUTED									
		1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120	
<i>P. stewartii</i> (8).....	B-108	++++	++	±	±	±	±	±	—	—	
<i>P. stewartii</i> (8).....	B-8	+	±	±	±	±	±	±	+	—	
<i>P. stewartii</i> (8).....	B-94	—	±	±	±	±	±	±	—	—	
<i>P. stewartii</i> (167).....	B-108	++++	++++	++	+	+	+	+	—	—	
<i>P. stewartii</i> (167).....	B-8	+++	++	+	+	+	+	+	—	—	
<i>P. stewartii</i> (167).....	B-94	±	+	+	+	+	±	—	—	—	
<i>P. insidiosus</i> (B-33).....	B-108	—	—	—	—	—	—	—	+	—	
<i>P. insidiosus</i> (B-33).....	B-8	—	—	—	—	—	—	—	—	—	
<i>P. insidiosus</i> (B-33).....	B-94	—	—	—	—	—	—	—	—	—	
<i>P. insidiosus</i> (B-34).....	B-108	—	—	—	—	—	—	—	—	—	
<i>P. insidiosus</i> (B-34).....	B-8	—	—	—	—	—	—	—	—	—	
<i>P. insidiosus</i> (B-34).....	B-94	—	—	—	—	—	—	—	—	—	
<i>P. michiganensis</i> (B-51A).....	B-108	—	—	—	—	—	—	—	—	—	
<i>P. michiganensis</i> (B-51A).....	B-8	—	—	—	—	—	—	—	—	—	
<i>P. michiganensis</i> (B-51A).....	B-94	—	—	—	—	—	—	—	—	—	
<i>P. michiganensis</i> (631).....	B-108	—	—	—	—	—	—	—	—	—	
<i>P. michiganensis</i> (631).....	B-8	—	—	—	—	—	—	—	—	—	
<i>P. michiganensis</i> (631).....	B-94	—	—	—	—	—	—	—	—	—	
<i>P. solanaceara</i> (Tom).....	B-108	—	—	—	—	—	—	—	—	—	
<i>P. solanaceara</i> (Tom).....	B-8	—	—	—	—	—	—	—	—	—	
<i>P. solanaceara</i> (Tom).....	B-94	—	—	—	—	—	—	—	—	—	
<i>P. solanaceara</i> (Tob).....	B-108	—	—	—	—	—	—	—	—	—	
<i>P. solanaceara</i> (Tob).....	B-8	—	—	—	—	—	—	—	—	—	
<i>P. solanaceara</i> (Tob).....	B-94	—	—	—	—	—	—	—	—	—	
<i>P. sepedonicum</i> (105).....	B-108	—	—	—	—	—	—	—	—	—	
<i>P. sepedonicum</i> (105).....	B-8	—	—	—	—	—	—	—	—	—	
<i>P. sepedonicum</i> (105).....	B-94	—	—	—	—	—	—	—	—	—	
<i>P. sepedonicum</i> (106).....	B-108	—	—	—	—	—	—	—	—	—	
<i>P. sepedonicum</i> (106).....	B-8	—	—	—	—	—	—	—	—	—	
<i>P. sepedonicum</i> (106).....	B-94	—	—	—	—	—	—	—	—	—	
<i>P. flaccumfaciens</i> (B-43A).....	B-108	—	—	—	—	—	—	—	—	—	
<i>P. flaccumfaciens</i> (B-43A).....	B-8	—	—	—	—	—	—	—	—	—	
<i>P. flaccumfaciens</i> (B-43A).....	B-94	—	—	—	—	—	—	—	—	—	
<i>Motile bacterium</i> (470).....	B-108	—	—	—	—	—	—	—	—	—	
<i>Motile bacterium</i> (470).....	B-8	—	—	—	—	—	—	—	—	—	
<i>Motile bacterium</i> (470).....	B-94	—	—	—	—	—	—	—	—	—	
<i>Motile bacterium</i> (480).....	B-108	—	—	—	—	—	—	—	—	—	
<i>Motile bacterium</i> (480).....	B-8	—	—	—	—	—	—	—	—	—	
<i>Motile bacterium</i> (480).....	B-94	—	—	—	—	—	—	—	—	—	
<i>P. stewartii</i> (B-110).....	B-108	++	++	++++	++	++	+	±	—	—	
<i>P. stewartii</i> (B-110).....	B-8	+	±	+	+	+	±	—	—	—	
<i>P. stewartii</i> (B-110).....	B-94	++	++	+	+	+	±	—	—	—	
<i>P. stewartii</i> (B-111).....	B-108	?	?	?	?	?	—	—	—	—	
<i>P. stewartii</i> (B-111).....	B-8	?	?	?	?	?	—	—	—	—	
<i>P. stewartii</i> (B-111).....	B-94	?	?	?	?	?	—	—	—	—	
<i>P. stewartii</i> (B-112).....	B-108	+	+	++	+	—	—	—	—	—	
<i>P. stewartii</i> (B-112).....	B-8	+	+	+	—	—	—	—	—	—	
<i>P. stewartii</i> (B-112).....	B-94	±	+	+	±	—	—	—	—	—	
<i>P. stewartii</i> (B-113).....	B-108	+	+	++	+	++	±	—	—	—	
<i>P. stewartii</i> (B-113).....	B-8	++	+	++	++	++	+	—	—	—	
<i>P. stewartii</i> (B-113).....	B-94	+	++	++	++	++	+	—	—	—	
<i>P. stewartii</i> (B-114).....	B-108	+	±	±	—	—	—	—	—	—	
<i>P. stewartii</i> (B-114).....	B-8	±	—	—	—	—	—	—	—	—	
<i>P. stewartii</i> (B-114).....	B-94	—	±	—	—	—	—	—	—	—	
<i>P. stewartii</i> (B-115).....	B-108	+	+	+	±	±	—	—	—	—	
<i>P. stewartii</i> (B-115).....	B-8	++	+	+	+	+	±	—	—	—	
<i>P. stewartii</i> (B-115).....	B-94	+	+	+	+	+	+	—	—	—	
<i>P. stewartii</i> (B-116).....	B-108	+	++	++	±	—	—	—	—	—	
<i>P. stewartii</i> (B-116).....	B-8	+	+	+	±	—	—	—	—	—	
<i>P. stewartii</i> (B-116).....	B-94	+	+	+	+	—	—	—	—	—	
<i>P. stewartii</i> (B-117).....	B-108	+	++	+	+	+	+	±	—	—	
<i>P. stewartii</i> (B-117).....	B-8	+++	+++	++	++	+	+	—	—	—	
<i>P. stewartii</i> (B-117).....	B-94	+	+	+	+	+	+	—	—	—	
<i>P. stewartii</i> (B-118).....	B-108	++++	++++	++	+	+	+	±	—	—	
<i>P. stewartii</i> (B-118).....	B-8	+	+	+	+	+	+	±	±	—	
<i>P. stewartii</i> (B-118).....	B-94	+	+	+	+	+	+	+	+	—	
<i>P. stewartii</i> (B-119).....	B-108	+	+	—	—	—	—	—	—	—	
<i>P. stewartii</i> (B-119).....	B-8	—	—	—	—	—	—	—	—	—	
<i>P. stewartii</i> (B-119).....	B-94	—	—	—	—	—	—	—	—	—	

\* None of the strains agglutinated when incubated in saline solution.

As a control on the potency of the three sera, two field isolates of *Phytomonas stewarti* and ten strains of *P. stewarti*—obtained from Dr. LINCOLN and numbered B-110 to B-119—were used. Strain B-110 was isolated at Hillsboro, Kentucky, in 1938. All the other strains were derived from a subculture of B-8, but they differed in virulence, colony characteristics, color, and viscosity. B-114, a moderately virulent white type, was not so strongly agglutinated as the other strains. Strain B-111, a mucoid type, formed strings of slime in the immune sera which prevented accurate observation on the extent of agglutination. As may be seen from table 6, the strains of *P. stewarti* were the only cultures to be agglutinated by the immune sera. The failure of the motile bacterium to agglutinate in immune serum brings additional evidence that it is not related to *P. stewarti*, even though it is capable of causing some wilt symptoms. It may be concluded that the agglutination test is probably specific for *P. stewarti*.

### Discussion

The agglutination reaction has been advocated by several plant pathologists (4, 10, 22) as an aid in identifying bacterial plant pathogens. ST. JOHN-BROOKS *et al.* (17) found that phytopathogenic bacteria could be grouped according to their agglutination reactions. GOLDSWORTHY (4, 5) used the test in identifying field isolates, while LINK and associates (9, 10, 11, 18) made use of reciprocal agglutination tests in determining the group relations of bacteria. Serological methods were widely accepted after it had been demonstrated that strains of *Phytomonas malvacearum* (E.F.S.) Bergey *et al.* (6, 26), *P. pisi* (Sack.) Bergey *et al.* (22), and *P. tabaca* (W. and F.) Bergey *et al.* (21) were identical with other strains of their respective species. STAPP (21) suggested that the two bacterial parasites of tobacco, *P. tabaca* and *P. angulata* (F. and M.) Bergey *et al.*, were the same because of their identical serological characteristics. This conclusion has since found support in the data presented by BRAUN (1) on the serological characteristics and interconversion of strains of the two bacteria.

Because of the growing tendency to rely upon serological methods in identifying bacteria, BURKHOLDER (2) has suggested that data on the reliability of the method were needed. The data presented in this paper show some of the limitations of the agglutination test in so far as it applies to *Phytomonas stewarti*. Strains may differ in their ability to agglutinate in immune sera and to incite agglutinin production in rabbits. In conducting agglutination tests for the identification of cultures, it would be necessary to use more than one serum, and even then the failure of a strain to agglutinate would not constitute evidence that it was not a strain of *P. stewarti*. The identity of such non-agglutinating strains would have to be established by some test other than serological behavior. On the other hand, there seems no good reason why agglutination in immune serum for *P. stewarti* should

not be accepted as evidence that the strain belongs in the species. At least the tests conducted with other closely related species failed to show any lack of specificity.

Apparently the serological characteristics of *P. stewarti* change very easily. For example, several of the variant strains isolated from B-11 were entirely different from the parent strain in their serological reactions. This ability of *P. stewarti* to produce new types probably explains the presence of serologically distinct strains in the same leaf lesion (table 2). It has been shown that strains of *P. tumefaciens* (E.F.S. and Town) Bergey *et al.* (19), *Erwinia carotovora* (Jones) Hol. (12, 20), and *Rhizobium* sp. (3, 23) isolated from different sources differ in their serological properties. A certain degree of stability for the soybean nodule bacterium was indicated by WRIGHT'S (28) observations that only one of four serological types could be isolated from any particular nodule and that the isolates did not change during incubation on culture media.

The differences in serological behavior of strains of *P. stewarti* may be due either to differences in antigenic composition or to the ease with which the strains agglutinate. If they possess different antigens they will absorb different types of agglutinins, but agglutinin-absorption tests would have to be made in order to detect these differences. Such tests have been made and will be reported later. It is of interest that the differences in serological behavior of the fifteen strains were not closely correlated with differences in virulence, colony characteristics, or physiological abilities. As a general rule the rough forms of bacteria are more easily agglutinated. B-8, which produced a dry crustaceous growth and approached a rough type, agglutinated very well but not appreciably better than some of the typical smooth types, such as B-102.

### Summary

1. The agglutination test was used in an attempt to identify ninety single-colony isolates of *Phytomonas stewarti* which were obtained from twenty-seven naturally infected maize plants at Princeton, New Jersey. Three of the isolates failed to agglutinate in either of two sera used, some agglutinated in one or the other but not in both, and the remainder agglutinated in both sera at dilutions of 1:20 to 1:2560. There was no consistent difference in the serological properties of isolates from susceptible and resistant inbred lines of corn. Some isolates from the same leaf lesion were as different from one another as were those from different plants.

2. Strains derived from the same culture were found to differ in their serological characteristics. A study of fifteen strains showed that the serological properties were not correlated with any particular colony characteristic, physiological ability, or degree of virulence. Strains from different localities in the United States and



Mexico were no more different from one another than some of the variants derived from one of the strains. Some strains agglutinated in all sera at titers as high as 1:1280 to 1:5120. These strongly agglutinating strains usually induced immune sera that failed to agglutinate five of the strains. The strains that agglutinated poorly, on the other hand, produced sera that were effective against all the cultures tested.

3. Several other species of wilt-producing bacteria did not agglutinate in anti-serum for three strains of *P. stewarti*.

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# AGGLUTININ ABSORPTION BY DIFFERENT STRAINS OF PHYTOMONAS STEWARTI

ARMIN C. BRAUN AND GEORGE L. MCNEW

## Introduction

In previous studies (5), strains of the sweet-corn wilt bacterium, *Phytophthora stewarti* (E. F. S.) Bergey *et al.*, were found to differ in their agglutination reactions. Some of the fifteen strains tested agglutinated readily in both homologous and heterologous immune serum, while others agglutinated poorly or not at all. As a general rule the cultures that agglutinated readily were poor antigens and produced an inferior type of immune serum. It was considered probable that the differences in the agglutination reactions resulted not alone from distinct serological differences in the strains but also from the inagglutinability of certain strains. Since the absorption of agglutinins by bacterial strains is generally recognized as a reliable serological method for determining relationships, reciprocal agglutinin-absorption tests were made for several of the agglutinable and inagglutinable strains. The results are presented in this paper.

## Material and methods

The sera and cultures used in previous tests (5) were employed. The cultures had been kept on nutrient-dextrose agar slants at 8° C. and the sera had been frozen and stored in an icebox. So far as could be determined, neither the cultures nor the sera had changed appreciably during the 2 months of storage under these conditions.

Since it was not feasible to run absorption tests on all the sera at once, only two or three sera and their homologous bacterial strains were used in each of a series of five tests. As a general rule each test included one of the strains that agglutinated readily, one that agglutinated poorly, and a third that was closely related to one of the other strains in that it was derived from the same culture but differed from it in virulence, cultural characteristics, or physiological abilities. The antisera for the different strains used in each test were thawed and divided into three samples consisting of 1 cc. each. Each sample was absorbed by one or the other of the three strains. As a control, a fourth sample of each serum was diluted in saline and kept by the side of the other three during the absorptions.

The absorptions were conducted according to routine methods. Bacteria were grown on nutrient-dextrose agar plates and suspended in sterile saline. Usually 3 or 4 cc. of concentrated bacterial suspension was introduced into each sample

of serum, thoroughly mixed by shaking, and incubated at 40° C. for 2 hours. The bacteria and their absorbed agglutinins were then centrifuged from the suspension and a fresh lot of bacteria added. After five or six such absorptions, when the sera had lost much of their potency, they were mixed with fresh lots of bacteria and stored overnight at 8° C. to complete the absorption. Such absorbed sera were centrifuged to remove as many bacteria as possible and sufficient saline added to give a final dilution of 1:10 or 1:20.

The absorbed sera and their unabsorbed controls were divided into 1 cc. samples, diluted in saline, and tested for agglutinins by the methods described previously (5). Samples of the unabsorbed sera were tested against the three strains used in that particular test. The absorbed sera were tested against all fifteen strains except where otherwise noted in the text. In tests where the absorbed sera had been cleared of agglutinins, only the data on the three strains used in that particular test are reported.

### Experimental results

Strains B-1611, B-1111, and B-91 and their immune sera were used in the first test. B-91 was a highly virulent type that agglutinated poorly, while B-1611 and B-1111 were practically avirulent and agglutinated readily. B-1611 and B-1111 were derived from strain B-11 but differed in their physiological properties. B-1611 used inorganic nitrogen while B-1111 did not. Each of the three cultures absorbed the agglutinins from their own serum as well as from the sera for the other two cultures. The absorbed sera were tested for residual agglutinins. None of them caused agglutination, as may be seen from table 1. Since each strain absorbed the agglutinins produced by the other two, it may be concluded that all three were identical in their antigenic composition. The other twelve strains also tested against the absorbed sera were not agglutinated.

It is of interest that B-91, which failed to agglutinate in immune serum for B-1611 and which gave doubtful agglutination in the immune serum for B-1111, had absorbed all the agglutinins from these two sera. This strain apparently failed to agglutinate because it was resistant to the physical forces that cause agglutination of the bacterial cells. Further evidence of resistance to agglutination is brought out by the fact that B-91 agglutinated poorly in its own serum. Unless it be postulated that some kind of non-specific agglutinin absorption was involved, it must be concluded that strains B-91, B-1111, and B-1611 are of identical antigenicity in spite of the differences noted in table 1 and elsewhere (5) on their ability to agglutinate. The possibility of non-specific absorption seems rather remote in view of the results obtained with other strains in the third and fourth tests.

In the second test, strains B-102, B-96, and B-94 and their immune sera were

used. Strain B-102 agglutinated easily and had produced an immune serum that distinctly failed to agglutinate other strains such as B-94 and B-96. The other two strains agglutinated poorly but had produced fairly potent sera. Both B-96

TABLE 1  
REACTIONS OF THREE STRAINS OF *P. STEWARTI* IN ABSORBED AND UNABSORBED  
IMMUNE SERA FOR B-1611, B-1111, AND B-91\*

ANTI-SERUM FOR STRAIN	SERUM ABSORBED BY STRAIN	STRAIN TESTED	AGGLUTINATION OF BACTERIA IN SERUM DILUTED								
			1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120
B-1611	B-1611	B-1611	—	—	—	—	—	—	—	—	—
	"	B-1111	—	—	—	—	—	—	—	—	—
	"	B-91	—	—	—	—	—	—	—	—	—
	B-1111	B-1611	—	—	—	—	—	—	—	—	—
	"	B-1111	—	—	—	—	—	—	—	—	—
	"	B-91	—	—	—	—	—	—	—	—	—
	B-91	B-1611	—	—	—	—	—	—	—	—	—
	"	B-1111	—	—	—	—	—	—	—	—	—
	"	B-91	—	—	—	—	—	—	—	—	—
	Unabsorbed	B-1611	+	+	+	+	+	—	—	—	—
B-1111	"	B-1111	+	+	+	+	+	—	—	—	—
	"	B-91	—	—	—	—	—	—	—	—	—
	B-1611	B-1611	—	—	—	—	—	—	—	—	—
	"	B-1111	—	—	—	—	—	—	—	—	—
	"	B-91	—	—	—	—	—	—	—	—	—
	B-1111	B-1611	—	—	—	—	—	—	—	—	—
	"	B-1111	—	—	—	—	—	—	—	—	—
	"	B-91	—	—	—	—	—	—	—	—	—
	B-91	B-1611	—	—	—	—	—	—	—	—	—
	"	B-1111	—	—	—	—	—	—	—	—	—
B-91	"	B-91	—	—	—	—	—	—	—	—	—
	Unabsorbed	B-1611	±	+	+	+	+	+	+	+	—
	"	B-1111	±	+	+	+	+	—	—	—	—
	"	B-91	±	—	—	—	—	—	—	—	—
	B-1611	B-1611	—	—	—	—	—	—	—	—	—
	"	B-1111	—	—	—	—	—	—	—	—	—
	"	B-91	—	—	—	—	—	—	—	—	—
	B-1111	B-1611	—	—	—	—	—	—	—	—	—
	"	B-1111	—	—	—	—	—	—	—	—	—
	"	B-91	—	—	—	—	—	—	—	—	—
	B-91	B-1611	—	—	—	—	—	—	—	—	—
	"	B-1111	—	—	—	—	—	—	—	—	—
	"	B-91	—	—	—	—	—	—	—	—	—
	Unabsorbed	B-1611	+	+	+	+	+	—	—	—	—
	"	B-1111	+++	+++	+	+	+	+	—	—	—
	"	B-91	+++	+	—	—	—	—	—	—	—
	B-1611	B-1611	—	—	—	—	—	—	—	—	—
	"	B-1111	—	—	—	—	—	—	—	—	—
	"	B-91	—	—	—	—	—	—	—	—	—
	B-1111	B-1611	—	—	—	—	—	—	—	—	—
	"	B-1111	—	—	—	—	—	—	—	—	—
	"	B-91	—	—	—	—	—	—	—	—	—

\* None of the strains agglutinated when incubated in saline control.

and B-94 were obtained from strain B-8, but B-96 was highly virulent and B-94 avirulent. After the immune sera for these three strains had been absorbed five times by the homologous and heterologous cultures, they were tested against all fifteen strains for residual agglutinins. The data on these tests are not presented,

since each culture had absorbed all the agglutinins from the three sera. Apparently these strains were of identical antigenic composition.

Strains B-8 and B-11 were selected for the third test. These two cultures were characterized by the ease with which B-8 agglutinated at high titers in all immune sera used and by the poor agglutinability of B-11. The immune serum produced by B-11 was the most generally effective of those tested (5), while that of B-8 failed to agglutinate five of the fifteen strains, including B-11.

These two cultures proved to be distinctly different in their abilities to incite and absorb agglutinins. As shown in table 2, B-8 cleared the B-11 immune serum of agglutinins effective against itself without removing the agglutinins effective against B-11 and the other strains. In the reciprocal absorption, B-11 failed to remove all the agglutinins from the B-8 immune serum. The residual agglutinins were effective against B-8 and B-107. This suggests that a closer relationship existed between the New York (B-8) and Mexico (B-107) strains than between either of these and the New Jersey (B-11) strain. In addition to the specific agglutinins produced by each strain and not absorbed by the other, apparently each culture produced some agglutinins that were absorbed by the other. At least the cross-absorptions reduced the titer and intensity of agglutination in the two sera. This suggests that the two strains are similar in some respects even though they may be distinguished by certain specific differences.

The residual agglutinins in the absorbed sera do not appear to be the result of incomplete absorptions, because both cultures had cleared the heterologous sera of agglutinins effective against themselves. Furthermore, both had cleared their own homologous sera of agglutinins by an identical treatment. Of course, the failure of B-11 to agglutinate in absorbed B-8 serum cannot be accepted as evidence that all agglutinins had been removed, since this culture would not agglutinate even in unabsorbed serum. The failure of B-8 to show definite agglutination in absorbed B-11 serum gave conclusive evidence that agglutinins for this culture had been removed.

Strains B-1011a, B-107, and B-108 were used in the fourth test. The three strains were obtained from different geographical areas. B-1011a was avirulent while the other two were highly virulent. Reciprocal absorptions were made on the antiserum of each. After five absorptions, the sera were tested for residual agglutinins against all fifteen strains. The data obtained on the sera absorbed by different strains are presented in table 3. Where the sera had been cleared of agglutinins, only data on B-1011a, B-107, and B-108 are presented. The data on all fifteen strains are given only for those sera that had residual agglutinins.

Strains B-1011a and B-108 appeared identical in their antigenic composition, since each completely absorbed the agglutinins produced by the other. B-107 differed from the other two in ability to absorb agglutinins. It failed to clear B-108

TABLE 2

AGGLUTINATION OF *P. STEWARTI* CULTURES IN ABSORBED AND UNABSORBED  
IMMUNE SERA FOR CULTURES B-11 AND B-8\*

ANTI-SERUM FOR STRAIN	SERUM ABSORBED BY STRAIN	STRAIN TESTED	AGGLUTINATION OF BACTERIA IN SERUM DILUTED								
			1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120
B-11	B-8	B-11	+	++	++	+	-	-	-	-	-
	B-8	B-1311	++	++	+	-	-	-	-	-	-
	B-8	B-1011a	++	++	+	+	-	-	-	-	-
	B-8	B-1611	++	++	++	-	-	-	-	-	-
	B-8	B-1111	+++	+	-	-	-	-	-	-	-
	B-8	B-107	+	+	+	+	-	-	-	-	-
	B-8	B-108	++	+	+	+	-	-	-	-	-
	B-8	B-109	+	+	+	-	-	-	-	-	-
	B-8	B-102	++	+	+	+	±	-	-	-	-
	B-8	B-103	++	+	+	+	-	-	-	-	-
	B-8	B-8	±	-	-	-	-	-	-	-	-
	B-8	B-96	+++	++	+	-	-	-	-	-	-
	B-8	B-92	+	+	+	+	-	-	-	-	-
	B-8	B-91	+	+	+	+	-	-	-	-	-
	B-8	B-94	+++	+	+	+	-	-	-	-	-
B-11	B-11	B-11	-	-	-	-	-	-	-	-	-
	B-11	B-1311	-	-	-	-	-	-	-	-	-
	B-11	B-1011a	-	-	-	-	-	-	-	-	-
	B-11	B-1611	-	-	-	-	-	-	-	-	-
	B-11	B-1111	-	-	-	-	-	-	-	-	-
	B-11	B-107	-	-	-	-	-	-	-	-	-
	B-11	B-108	-	-	-	-	-	-	-	-	-
	B-11	B-109	-	-	-	-	-	-	-	-	-
	B-11	B-102	-	-	-	-	-	-	-	-	-
	B-11	B-103	-	-	-	-	-	-	-	-	-
	B-11	B-8	-	-	-	-	-	-	-	-	-
	B-11	B-96	-	-	-	-	-	-	-	-	-
	B-11	B-92	-	-	-	-	-	-	-	-	-
	B-11	B-91	-	-	-	-	-	-	-	-	-
	B-11	B-94	-	-	-	-	-	-	-	-	-
B-11	Unabsorbed	B-11	++++	+++	+++	±	-	-	-	-	-
	Unabsorbed	B-8	++	++	++	+	+	+	±	±	-
B-8	B-11	B-11	-	-	-	-	-	-	-	-	-
	B-11	B-1311	-	-	-	-	-	-	-	-	-
	B-11	B-1011a	-	-	-	-	-	-	-	-	-
	B-11	B-1611	-	-	-	-	-	-	-	-	-
	B-11	B-1111	-	-	-	-	-	-	-	-	-
	B-11	B-107	+	+	±	-	-	-	-	-	-
	B-11	B-108	-	-	-	-	-	-	-	-	-
	B-11	B-109	-	-	-	-	-	-	-	-	-
	B-11	B-102	±	-	-	-	-	-	-	-	-
	B-11	B-103	-	-	-	-	-	-	-	-	-
	B-11	B-8	+	+	+	+	-	-	-	-	-
	B-11	B-96	-	-	-	-	-	-	-	-	-
	B-11	B-92	-	-	-	-	-	-	-	-	-
	B-11	B-91	-	-	-	-	-	-	-	-	-
	B-11	B-94	-	-	-	-	-	-	-	-	-

\* None of the strains agglutinated when incubated in saline control.

TABLE 2—*Continued*

ANTI-SERUM FOR STRAIN	SERUM ABSORBED BY STRAIN	STRAIN TESTED	AGGLUTINATION OF BACTERIA IN SERUM DILUTED								
			1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120
B-8	B-8	B-11	—	—	—	—	—	—	—	—	—
	B-8	B-1311	—	—	—	—	—	—	—	—	—
	B-8	B-1011a	—	—	—	—	—	—	—	—	—
	B-8	B-1611	—	—	—	—	—	—	—	—	—
	B-8	B-1111	—	—	—	—	—	—	—	—	—
	B-8	B-107	—	—	—	—	—	—	—	—	—
	B-8	B-108	—	—	—	—	—	—	—	—	—
	B-8	B-109	—	—	—	—	—	—	—	—	—
	B-8	B-102	—	—	—	—	—	—	—	—	—
	B-8	B-103	—	—	—	—	—	—	—	—	—
	B-8	B-8	—	—	—	—	—	—	—	—	—
	B-8	B-96	—	—	—	—	—	—	—	—	—
	B-8	B-92	—	—	—	—	—	—	—	—	—
	B-8	B-91	—	—	—	—	—	—	—	—	—
B-8	B-94	—	—	—	—	—	—	—	—	—	
B-8	Unabsorbed	B-11	—	—	—	—	—	—	—	—	—
	Unabsorbed	B-8	++	+	+	+	+	+	+	—	—

antiserum of agglutinins for several other strains. On the other hand, B-1011a failed to clear B-107 antiserum of agglutinins. The fact that the residual agglutinins were effective against both B-107 and B-8 suggests that these two strains have something in common, as mentioned in discussing the data obtained in the third test.

In order to determine whether B-8 and B-107 would give comparable reactions when tested under similar conditions, a fifth experiment was made with these strains and B-11. Six absorptions were made with each strain on separate samples of the three homologous and heterologous sera, and then the sera were used for agglutination tests against strains B-107, B-8, B-11, B-102, B-1111, B-92, and B-94. The data confirm those reported in tables 2 and 3. B-11 failed to clear either B-8 or B-107 immune sera of agglutinins for these two strains. On the other hand, B-8 and B-107 failed to clear the antiserum for B-11 of agglutinins effective against the other strains.

The facts that B-11 failed to remove agglutinins from antisera for either B-107 or B-8, and that the residual agglutinins were effective against both strains, suggest that they are closely related. However, B-107 and B-8 left agglutinins in B-11 serum that were effective against each other. It follows that if the cultures were antigenically distinct, they would not completely absorb the agglutinins from their respective heterologous sera. As shown in table 4, however, they did completely absorb each other's agglutinins. The answer to this discrepancy in the results is not immediately obvious. In repetitions of the experiment it was observed that



TABLE 3

AGGLUTINATION OF *P. STEWARTI* CULTURES IN ABSORBED AND UNABSORBED  
IMMUNE SERA FOR B-1011a, B-107, AND B-108\*

ANTI-SERUM FOR STRAIN	SERUM ABSORBED BY STRAIN	STRAIN TESTED	AGGLUTINATION OF BACTERIA IN SERUM DILUTED								
			1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120
B-107	B-107	B-1011a	—	—	—	—	—	—	—	—	—
	B-107	B-107	—	—	—	—	—	—	—	—	—
	B-107	B-108	—	—	—	—	—	—	—	—	—
	B-1011a	B-11	—	—	—	—	—	—	—	—	—
	B-1011a	B-1311	—	—	—	—	—	—	—	—	—
	B-1011a	B-1011a	—	—	—	—	—	—	—	—	—
	B-1011a	B-1611	—	—	—	—	—	—	—	—	—
	B-1011a	B-1111	—	—	—	—	—	—	—	—	—
	B-1011a	B-107	+	+	±	±	—	—	—	—	—
	B-1011a	B-108	—	—	—	—	—	—	—	—	—
	B-1011a	B-109	—	—	—	—	—	—	—	—	—
	B-1011a	B-102	—	—	—	—	—	—	—	—	—
	B-1011a	B-103	—	—	—	—	—	—	—	—	—
	B-1011a	B-8	±	+	+	±	—	—	—	—	—
	B-1011a	B-96	—	—	—	—	—	—	—	—	—
	B-1011a	B-92	—	—	—	—	—	—	—	—	—
	B-1011a	B-91	—	—	—	—	—	—	—	—	—
	B-1011a	B-94	—	—	—	—	—	—	—	—	—
	B-108	B-11	—	—	—	—	—	—	—	—	—
	B-108	B-1311	—	—	—	—	—	—	—	—	—
	B-108	B-1011a	—	—	—	—	—	—	—	—	—
	B-108	B-1611	—	—	—	—	—	—	—	—	—
	B-108	B-1111	—	—	—	—	—	—	—	—	—
	B-108	B-107	—	—	—	—	—	—	—	—	—
	B-108	B-108	—	—	—	—	—	—	—	—	—
	B-108	B-109	—	—	—	—	—	—	—	—	—
	B-108	B-102	—	—	—	—	—	—	—	—	—
	B-108	B-103	—	—	—	—	—	—	—	—	—
	B-108	B-8	+	+	+	+	—	—	—	—	—
	B-108	B-96	—	—	—	—	—	—	—	—	—
	B-108	B-92	—	—	—	—	—	—	—	—	—
	B-108	B-91	—	—	—	—	—	—	—	—	—
	B-108	B-94	—	—	—	—	—	—	—	—	—
	Unabsorbed	B-1011a	—	—	—	—	—	—	—	—	—
	Unabsorbed	B-107	±	+	+	+	+	+	±	—	—
	Unabsorbed	B-108	—	±	±	—	—	—	—	—	—
B-1011a	B-1011a	B-1011a	—	—	—	—	—	—	—	—	—
	B-1011a	B-107	—	—	—	—	—	—	—	—	—
	B-1011a	B-108	—	—	—	—	—	—	—	—	—
	B-107	B-1011a	—	—	—	—	—	—	—	—	—
	B-107	B-107	—	—	—	—	—	—	—	—	—
	B-107	B-108	—	—	—	—	—	—	—	—	—
	B-108	B-1011a	—	—	—	—	—	—	—	—	—
	B-108	B-107	—	—	—	—	—	—	—	—	—
	B-108	B-108	—	—	—	—	—	—	—	—	—
	Unabsorbed	B-1011a	+++	+	—	—	—	—	—	—	—
	Unabsorbed	B-107	+	+	+	±	±	—	—	—	—
	Unabsorbed	B-108	+	+	—	—	+	—	—	—	—

\* None of the strains agglutinated when incubated in saline control.

TABLE 3—Continued

ANTI-SERUM FOR STRAIN	SERUM ABSORBED BY STRAIN	STRAIN TESTED	AGGLUTINATION OF BACTERIA IN SERUM DILUTED								
			1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120
B-108	B-108	B-1011a	—	—	—	—	—	—	—	—	—
	B-108	B-107	—	—	—	—	—	—	—	—	—
	B-108	B-108	—	—	—	—	—	—	—	—	—
	B-107	B-11	—	±	—	—	—	—	—	—	—
	B-107	B-1311	—	—	—	—	—	—	—	—	—
	B-107	B-1011a	—	—	—	—	—	—	—	—	—
	B-107	B-1611	—	±	—	—	—	—	—	—	—
	B-107	B-1111	+	+	+	+	—	—	—	—	—
	B-107	B-107	—	—	—	—	—	—	—	—	—
	B-107	B-108	±	+	±	—	—	—	—	—	—
	B-107	B-109	—	—	—	—	—	—	—	—	—
	B-107	B-102	—	—	—	—	—	—	—	—	—
	B-107	B-103	±	±	—	—	—	—	—	—	—
	B-107	B-8	+	+	±	—	—	—	—	—	—
	B-107	B-96	—	+	±	±	—	—	—	—	—
	B-107	B-92	±	+	+	±	±	—	—	—	—
	B-107	B-91	—	±	+	—	—	—	—	—	—
	B-107	B-94	—	—	—	—	—	—	—	—	—
	B-1011a	B-1011a	—	—	—	—	—	—	—	—	—
	B-1011a	B-107	—	—	—	—	—	—	—	—	—
B-1011a	B-108	—	—	—	—	—	—	—	—	—	
Unabsorbed	B-1011a	+++	++	+	±	—	—	—	—	—	
Unabsorbed	B-108	++	+	+	—	—	—	—	—	—	
Unabsorbed	B-107	++	++	+	+	+	±	—	—	—	

some residual agglutinins were present in immune sera for B-107 and B-8 after they had been absorbed by the heterologous cultures. The amount of residual agglutinins was so small that their effects could be detected only by microscopic examination of the bacterial suspension in the absorbed sera. Small clumps of 3-30 cells could be detected, particularly in B-8 immune serum absorbed by B-107 and tested against B-8. Similar clumps were not observed in the immune sera that had been absorbed by their homologous strains. It is believed, therefore, that B-107 and B-8 are not identical, even though they are very similar in most of their antigenic properties. The agglutinins which each failed to absorb from antiserum of the other were a minor fraction of the total.

### Discussion

Many strains of *Phytomonas stewarti* have been identified in recent years (1, 2, 3, 4, 6, 7) by differences in their virulence, colony characteristics, color, or physiological abilities. According to IVANOFF *et al.* (1), at least three major types exist under field conditions. In view of these differences, it is not surprising that some strains should also differ in their antigenic composition. No correlation could be detected,

TABLE 4  
AGGLUTINATION OF *P. STEWARTI* IN ABSORBED IMMUNE SERA  
OF CULTURES B-II, B-107, AND B-8\*

ANTISERUM FOR STRAIN	SERUM ABSORBED BY STRAIN	STRAIN TESTED	AGGLUTINATION OF BACTERIA IN IMMUNE SERUM DILUTED					
			1:10	1:20	1:40	1:80	1:160	1:320
B-II	B-107	B-107	—	—	—	—	—	—
	B-107	B-II	+	+	++	+	+	±
	B-107	B-8	+	+	+	+	+	±
	B-107	B-102	+	+	+	+	+	+
	B-107	B-IIII	±	±	±	±	—	—
	B-107	B-92	±	+	—	—	—	—
	B-107	B-94	—	—	—	—	—	—
B-II	B-8	B-107	+++	+	—	—	—	—
	B-8	B-II	+++	+	—	—	—	—
	B-8	B-8	—	—	—	—	—	—
	B-8	B-102	±	+	+	+	—	—
	B-8	B-IIII	—	—	—	—	—	—
	B-8	B-92	+	+	+	+	—	—
	B-8	B-94	+	+	+	+	—	—
B-II	B-II	B-107	—	—	—	—	—	—
	B-II	B-II	—	—	—	—	—	—
	B-II	B-8	—	—	—	—	—	—
B-II	Unabsorbed	B-107	.....	+++	+++	+++	++	++
	Unabsorbed	B-II	.....	+++++	+++++	+++++	±	±
	Unabsorbed	B-8	.....	++	++	++	+	+
B-107	B-8	B-107	—	—	—	—	—	—
	B-8	B-II	—	—	—	—	—	—
	B-8	B-8	—	—	—	—	—	—
	B-8	B-102	—	—	—	—	—	—
	B-8	B-IIII	—	—	—	—	—	—
	B-8	B-92	—	—	—	—	—	—
	B-8	B-94	—	—	—	—	—	—
B-107	B-II	B-107	+	+	+	+	—	—
	B-II	B-II	—	—	—	—	—	—
	B-II	B-8	+	+	+	±	—	—
	B-II	B-102	—	—	—	—	—	—
	B-II	B-IIII	—	—	—	—	—	—
	B-II	B-92	—	—	—	—	—	—
	B-II	B-94	—	—	—	—	—	—
B-107	B-107	B-107	—	—	—	—	—	—
	B-107	B-II	—	—	—	—	—	—
	B-107	B-8	—	—	—	—	—	—
B-107	Unabsorbed	B-107	.....	+	++	++	++	++
	Unabsorbed	B-II	.....	±	—	—	—	—
	Unabsorbed	B-8	.....	+	+	+	+	+

\* None of the strains agglutinated when incubated in saline control.

TABLE 4—Continued

ANTISERUM FOR STRAIN	SERUM ABSORBED BY STRAIN	STRAIN TESTED	AGGLUTINATION OF BACTERIA IN IMMUNE SERUM DILUTED					
			1:10	1:20	1:40	1:80	1:160	1:320
B-8	B-107	B-107	—	—	—	—	—	—
	B-107	B-11	—	—	—	—	—	—
	B-107	B-8	—	—	—	—	—	—
	B-107	B-102	—	—	—	—	—	—
	B-107	B-1111	—	—	—	—	—	—
	B-107	B-92	—	—	—	—	—	—
	B-107	B-94	—	—	—	—	—	—
B-8	B-11	B-107	+	+	+	—	—	—
	B-11	B-11	—	—	—	—	—	—
	B-11	B-8	+	+	+	+	±	—
	B-11	B-102	—	—	—	—	—	—
	B-11	B-1111	—	—	—	—	—	—
	B-11	B-92	—	—	—	—	—	—
	B-11	B-94	—	—	—	—	—	—
B-8	B-8	B-107	—	—	—	—	—	—
	B-8	B-11	—	—	—	—	—	—
	B-8	B-8	—	—	—	—	—	—
B-8	Unabsorbed	B-107	.....	+	+	+	+	±
	Unabsorbed	B-11	.....	—	—	—	—	—
	Unabsorbed	B-8	.....	++	++	+	+	+

however, between antigenic composition and other characteristics. For example, in some species of bacteria rough and smooth types are serologically distinct. In *P. stewartii* the firm crustaceous B-8 was very similar to the soft spreading B-107, and both were distinctly different from the smooth B-11. There was also no correlation between virulence and any particular antigenic type. Avirulent B-94 and highly virulent B-96 and B-102 were found to be closely related, while highly virulent B-11 and B-107 proved to be distinctly different in their serological characteristics.

The differences previously reported (5) on the agglutinability of the strains in immune serum can be interpreted in view of the present data. Some of the strains do produce and absorb specific agglutinins; but these specific types are only a fraction of the total and are not the major factor responsible for differences in agglutination. For example, B-94 failed to agglutinate in B-102 immune serum yet it absorbed the agglutinins just as effectively as did B-102, which agglutinated readily. Similarly, B-11 failed to agglutinate in the immune serum of B-8 in spite of the fact that it absorbed all the agglutinins except a minor type specialized for B-8. Conversely, B-8 agglutinated readily in B-11 immune serum even though it absorbed only a fraction of the agglutinins. Such differences in agglutinability might well be attributed to the resistance of the different strains to the physical

factors that cause agglutination after the agglutinins have been absorbed. These differences might have been anticipated because it is known that some strains of other species differ in the ease with which they agglutinate in saline solution. It follows that the simple agglutination test is inadequate in determining differences in the antigenic constituents of bacteria.

### Summary

1. Strains of *Phytomonas stewarti* that differed in virulence, colony type, physiology, and agglutination reaction were tested for ability to absorb agglutinins from immune sera. All the strains tested absorbed most of the agglutinins from both homologous and heterologous sera, indicating that they were similar in most essentials. The failure of some strains to agglutinate after absorbing agglutinins was apparently due to their resistance to this reaction.

2. Some differences were observed between the strains from Mexico (B-107), New York (B-8), and New Jersey (B-11). The strains from Mexico and New York failed to absorb all the agglutinins from the antiserum for the New Jersey strain. In reciprocal tests, the New Jersey strain failed to absorb all the agglutinins from immune sera of the other two strains.

3. The differences in serological properties were not closely correlated with any other characteristic, such as virulence or colony type.

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# NITROGEN METABOLISM OF THE PLANT EMBRYO

W. L. MCRARY

(WITH FOUR FIGURES)

## Introduction

In connection with recent studies on the protein metabolism of grasses and legumes, a new system of microanalysis for protein and its degradation products was developed by BORSOOK and DUBNOFF (2). Using these methods, these workers found that both grasses and legumes synthesized protein in the growing regions during germination, both in the light and darkness. Since such plants are relatively high in protein and carbohydrate reserves, it seemed of interest to investigate a plant belonging to the high-protein low-carbohydrate reserve class (Prianischnikow's third type). The present study deals with the lupin, a typical example of the latter group.

Morphologically the bulk of the lupin seed consists of two cotyledons which contain the reserve food supply. During germination the protein or its decomposition products are translocated downward from the cotyledons into the developing stem and root, and used in forming these new tissues. By analyzing separately the cotyledons and the stem and root (which together will be called the axis) at various stages of development, it is possible to follow the course of the nitrogen metabolism with regard to the site as well as to the nature of the changes involved.

Treatment of the tissue with hot water or alcohol, or drying or freezing, tends to change the original form of the nitrogenous constituents (4). Proteins and amides are especially labile to such treatment, and since these compounds were of particular interest in this study, less drastic treatment was sought. The method finally adopted consisted of repeated extractions and grinding of the fresh material with dilute  $\text{Na}_2\text{SO}_4$  solution, which dissolves both albumins and globulins as well as the less highly organized nitrogen compounds. By this method "insoluble nitrogen," the nature of which is different in each case according to the technique of extraction used, was reduced to less than 5 per cent of the total nitrogen. Determinations on such a complete extract can then serve better to identify the various forms of nitrogen, as well as to indicate more clearly the relationships between them.

## Experimentation

In a series of initial experiments, several nitrogen-free inorganic salt mixtures were tested as culture media. Analysis of the plants after 14 days' germination, however, showed that these media had no detectable effect on the nitrogen metabolism as compared with water controls, hence redistilled water alone was used.

The seeds used were the Hartwegii species of dark blue lupin, *Lupinus hartwegii*, obtained from the Ferry-Morse Seed Company. About one hundred seeds were soaked in redistilled water for 3 hours, then placed individually in vials containing water-saturated cotton. The vials were placed in a large glass-covered box in a constant temperature darkroom (25° C.) free from laboratory fumes. A large open vessel of water in the box prevented excess evaporation of moisture from the cotton. Continuous illumination was provided by a 100-watt lamp 18 inches above the vials. The etiolated plants received the same treatment except for the illumination.

At the end of the period of germination, the embryos were separated from the cotton, the seedcoats removed, and excess moisture taken up with filter paper. The cotyledons were cut off at their junction with the axis, and axis and cotyledons weighed and handled separately in all subsequent treatment.

The tissue from fifty plants was placed in a small heavy-walled test tube fitted to receive a glass grinding rod. A small amount of washed sand was added and the tissue ground to a fine pulp. One ml. of 4 per cent  $\text{Na}_2\text{SO}_4$  solution was added and the grinding continued another minute. The mixture was centrifuged and the supernatant transferred to a 10-ml. volumetric flask. This process was repeated until the supernatant was clear; the extract was then made to volume and the nitrogen determinations carried out by the method of BORSOOK and DUBNOFF. Water content was determined by the difference in weight of a sample of the tissue before and after drying at 100° C. for 10 hours. In addition to the nitrogen fractions shown in the following section, ammonia nitrogen was determined by micro distillation and peptide nitrogen by the use of a peptidase mixture described by ORCUTT and WILSON (4).

### Results

The results are expressed graphically in figures 1-4, in which the various forms of nitrogen (as percentage of total plant nitrogen) are plotted against the time in days. The total nitrogen in one hundred plants averaged 102 mg. The concentrations of amide and amino nitrogen in the water of the tissues are given in tables 1-4.

#### PLANTS GROWN IN DARKNESS

Figures 1 and 2 show that nitrogen is transferred from the cotyledons to the axis for about the first 12 days. This increase in total axis nitrogen is paralleled closely by a decrease in the protein nitrogen of the cotyledon. The protein nitrogen of the axis and the amino and amide nitrogen of the cotyledon show relatively little change during the period studied.

From the values given in table 1 it is seen that the concentration of amino nitrogen, and hence of amino acids, remains fairly constant in the axis, dropping to a

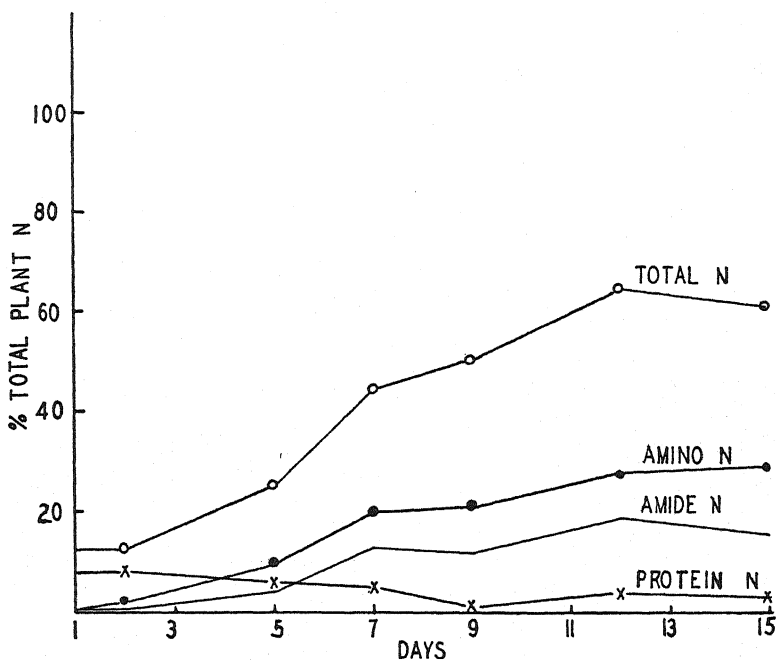


FIG. 1.—Etiolated series: Nitrogen distribution in axis

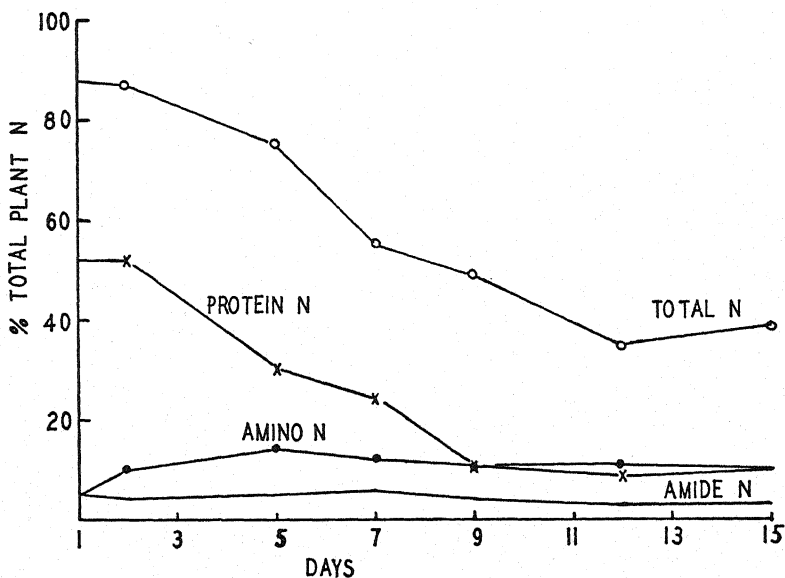


FIG. 2.—Etiolated series: Nitrogen distribution in cotyledons



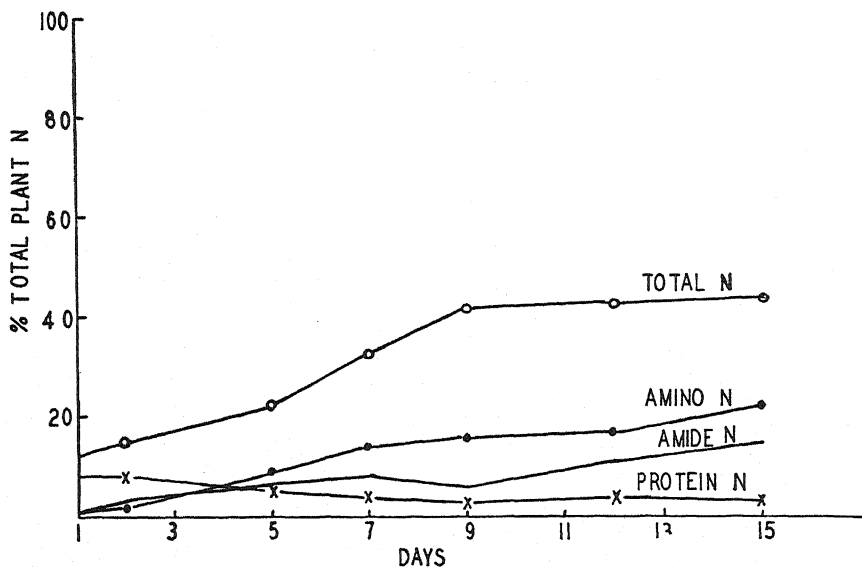


FIG. 3.—Illuminated series: Nitrogen distribution in axis

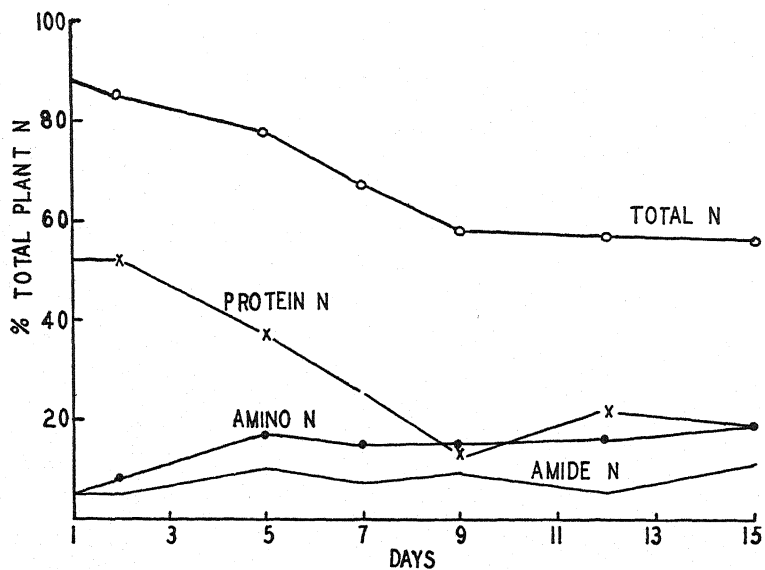


FIG. 4.—Illuminated series: Nitrogen distribution in cotyledons

TABLE 1  
MOLAL CONCENTRATION OF NITROGEN FRACTIONS IN WATER OF  
TISSUE. AXIS; ETIOLATED SERIES

NITROGEN	TIME IN DAYS						
	1	2	5	7	9	12	15
Amino.....	0.19	0.17	0.23	0.14	0.16	0.16	0.11
Amide.....	0.09	0.05	0.09	0.09	0.09	0.11	0.06

TABLE 2  
MOLAL CONCENTRATION OF NITROGEN FRACTIONS IN WATER OF  
TISSUE. COTYLEDONS; ETIOLATED SERIES

NITROGEN	TIME IN DAYS						
	1	2	5	7	9	12	15
Amino.....	0.18	0.34	0.35	0.25	0.27	0.24	0.19
Amide.....	0.16	0.14	0.13	0.13	0.09	0.08	0.06

TABLE 3  
MOLAL CONCENTRATION OF NITROGEN FRACTIONS IN WATER OF  
TISSUE. AXIS; ILLUMINATED SERIES

NITROGEN	TIME IN DAYS						
	1	2	5	7	9	12	15
Amino.....	0.19	0.16	0.29	0.14	0.16	0.16	0.16
Amide.....	0.09	0.21	0.16	0.09	0.06	0.10	0.11

TABLE 4  
MOLAL CONCENTRATION OF NITROGEN FRACTIONS IN WATER OF  
TISSUE. COTYLEDONS; ILLUMINATED SERIES

NITROGEN	TIME IN DAYS						
	1	2	5	7	9	12	15
Amino.....	0.18	0.23	0.34	0.23	0.17	0.16	0.18
Amide.....	0.16	0.14	0.19	0.11	0.10	0.05	0.10

somewhat lower value by the fifteenth day. Corresponding values for the amino nitrogen in the cotyledons are at a higher level. With the exception of the irregularity on the second day, the amide concentration in the axis remains constant up to the twelfth day, after which it drops. The amide concentration in the cotyledons decreases steadily throughout the period studied.

#### PLANTS GROWN IN LIGHT

The data for the axis are shown in figure 3. The increase in total nitrogen is less rapid than in the dark, and reaches a constant value by the ninth day. At this time the plant has attained its maximum growth in height (4.6-4.8 cm.). The amino and amide nitrogen values for the axis increase throughout this period, while the protein content shows little change.

The nitrogen changes in the cotyledons are represented in figure 4. Total and protein nitrogen decrease simultaneously until the ninth day; the protein then increases somewhat, possibly at the expense of amide nitrogen, which decreases slightly at this time. After the fifth day the amino nitrogen content is constant.

Tables 3 and 4 give the data for the concentration of amino and amide nitrogen in the water of the tissues. For the axis, the amino nitrogen concentration is fairly constant, with the exception of the value on the fifth day. The other values are too irregular to be of much significance.

In all the experiments ammonia nitrogen never exceeded 2 per cent of the total, and in most cases was much less. The peptide nitrogen in the axis constituted about 2 per cent of the total nitrogen in both light and dark series. In the cotyledons of the dark series the peptide nitrogen constituted 10 per cent of the total nitrogen for the first 5 days, then decreased to less than 2 per cent by the ninth day. In the illuminated series the peptide nitrogen fell to less than 2 per cent of the total nitrogen by the fifth day.

#### Discussion

BONNET (1), in a study of the nitrogen metabolism of *Lupinus luteus* in the very early stages of germination, reported significant increases in amino and amide nitrogen when the shoot just appeared (on the second day under the conditions reported in this paper). He gave no data on the subsequent changes occurring in axis and cotyledon.

McKIE (3) studied the changes occurring in lupins grown in a soil-sand mixture up to 25 days. She reported a minimum value for protein nitrogen on the eighteenth day, and decreasing values for amino and amide nitrogen from the eighth day onward. The asparagine content rose to a maximum of 53 per cent of the total nitrogen on the eighteenth day under her conditions of culture and analysis, and in all experiments amino and amide nitrogen was very low as compared with pro-

tein and "insoluble" nitrogen. The relations of "protein" and "insoluble" nitrogen to each other and to the other nitrogenous constituents are usually ill-defined unless the nature of the insoluble fraction is known, which is generally not the case.

Figures 1 and 2 (dark series) show that the amino and amide nitrogen of the cotyledon changed but little after the fifth day, whereas the protein content continued to decrease until the ninth day; concomitant with this protein decrease, the amide and amino nitrogen contents of the axis increased in close parallelism, suggesting a quantitative relationship among these three constituents. It should be noted that after the ninth day the protein content of the cotyledon changed very little but the amino acids of the axis continued to increase, suggesting synthesis of this nitrogen fraction.

With regard to the plants grown in the light, it appeared that illumination either promoted protein synthesis or inhibited its hydrolysis after the ninth day, as the protein content of the cotyledon was at a higher level in the illuminated series as compared with the etiolated plants. A similar condition was found by VICKERY *et al.* (5) to occur in the excised tobacco leaf after 73 hours. From the ninth to the fifteenth day a reciprocal relation existed between amide and protein in the cotyledon. The "protein sparing" action of light may be due to the fact that the illuminated plant obtains its energy for growth by the oxidation of carbohydrate synthesized in the presence of light, whereas the etiolated plant hydrolyzes protein to supply some of the required energy, unless it has adequate carbohydrate reserves. It is possible that under the conditions of these experiments photosynthesis cannot supply carbohydrate for energy purposes until the ninth day.

Associated with the increased protein content of the illuminated plants were definite morphological differences; thus the etiolated plants had small yellow cotyledons and tall thin stems (8.5 cm. in 15 days) whereas the illuminated plants had large green cotyledons and short sturdy stems (4.7 cm. in 15 days). These relations indicate that longitudinal growth and protein synthesis are not necessarily associated, and that even a reciprocal relation may hold.

Attention has already been called to the constancy of the values for amide and amino nitrogen concentration in the water of the tissues. Although these concentrations are irregular, they are of the same order of magnitude for each constituent throughout the period studied. This suggests that a dynamic relation exists between protein hydrolysis and the concentration of the soluble hydrolytic products.

### Summary

1. Data are presented on the values and distribution of the chief nitrogenous constituents of the lupin plant at progressive stages of germination.
2. Protein disappearing from the cotyledon was found to accumulate in the axis as amino acids and amides.

3. Plants grown in the dark were found to hydrolyze more protein and transport the products to the axis sooner than did plants grown in the light.
4. Longitudinal plant growth may take place to a marked extent with little or no change in the actual protein content of the elongating part.
5. The amino and amide nitrogen concentrations in the water of the lupin tissues remain at the same order of magnitude throughout the first 15 days of germination, suggesting a dynamic relation between these two nitrogen fractions and protein hydrolysis.

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# STARCH HYDROLYSIS IN BEAN LEAVES FOLLOWING SPRAYING WITH ALPHA NAPHTHALENE ACETIC ACID EMULSION

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(WITH FOUR FIGURES)

## Introduction

In previous investigations concerning the response of plants to applications of growth substances such as indoleacetic, naphthalene acetic, and indolebutyric acids, it was noted that starch in tissues near the treated region was hydrolyzed following treatment, and that although carbohydrate was in some cases mobilized in the treated region of stems, it did not occur as starch. The disappearance of starch from the region of bean and tomato stems treated with a mixture of indoleacetic acid and lanolin has been reported (1, 3). Other investigations have shown that starch was not present in the region of the stems of bean plants to which indoleacetic, naphthalene acetic, or indolebutyric acids were applied (3, 4, 5, 6). These responses suggested that the presence of these acids possibly increased the diastatic activity of enzymes in some cells that responded to treatment. In the present investigation a more direct study of the effect of some growth regulating substances on the rate at which starch was digested during a period of low light intensity or in darkness was made; also the rates of sugar accumulated in treated and control plants during a period of illumination were compared.

To facilitate the study, treated and control leaves of bean plants were grown: (a) in alternate periods of natural daylight and darkness, (b) in complete darkness, and (c) in natural daylight. The starch and sugar content of treated and control leaves under these various conditions was determined quantitatively.

## Methods and preliminary experiments

The heart-shaped leaves of kidney bean plants, Calaprooted variety, were used. The plants were grown in soil in 4-inch clay pots, an excess being planted so that uniform plants could be selected for each experiment. In experiments in which attached leaves were required, all portions of the plants above the second node were removed previous to treatment so as to accelerate the accumulation of carbohydrates in the primary leaves and to prevent further extension of the stem or production of additional leaves.

In preliminary experiments, detached leaves of bean plants were rooted in

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sand kept moist with complete nutrient, and exposed to natural daylight until they contained a relatively large amount of starch. Of some of these the entire upper surfaces were painted with a warm 2 per cent lanolin mixture of naphthalene acetic acid; of others only portions were painted in a similar way; still others were treated in the same manner with pure lanolin, as controls. One week later the chlorophyll was removed by means of hot alcohol and the leaf blades immersed in weak iodine solution. A strong positive starch test was observed in control leaves, while leaves treated with the lanolin-naphthalene acetic acid mixture showed in most cases a much less deposit of starch in the treated areas. In some few instances these treated areas gave no positive starch test. The results indicated that—other conditions being the same—more starch was hydrolyzed in areas of the leaves to which the lanolin-naphthalene acetic acid mixture had been applied than in those to which only lanolin was applied. Leaves still attached to the plants were treated and found to respond in a similar manner. Naphthalene acetic acid in a lanolin water emulsion was then tried and this was found to be a more suitable method of application than had been used. Application of an emulsion containing 1 per cent naphthalene acetic acid resulted in increased hydrolysis of starch in leaves without noticeable toxicity, and this concentration was chosen for further experimentation.

The following method for making emulsions was adopted. Two hundred and fifty mg. of alpha naphthalene acetic acid was thoroughly mixed in 25 gm. of melted lanolin. The mixture was added to 250 ml. of boiling water in which 1.25 gm. of laundry soap and 0.2 gm. of agar had been dissolved. The mixture was thoroughly stirred, cooled, and applied to the leaves by means of a spray, using a paper collar around each petiole, just below the leaf blade, to protect the remainder of the plant from the spray.

For chemical analysis, duplicate samples consisting of twenty-four leaves each were collected, dried at 80° C. in a well ventilated oven, and ground to 100 mesh. They were redried at 80° C. in a vacuum. The sugar, and the combined starch and dextrin contained in 1-gm. samples, were determined as previously described (5). Sucrose was hydrolyzed by means of invertase and the total amount of reducing and non-reducing sugars determined.

### Experimental results

A series of three experiments was conducted. The object of the first experiment was to determine whether the starch, dextrin, and sugar content of sprayed leaves varied from that of control leaves when the plants were grown in alternate periods of natural daylight and darkness subsequent to treatment.

Plants having leaves 2-3 inches in width and containing appreciable amounts of starch, as shown by iodine tests, were used. Several hundred plants were

selected for size and uniformity and placed in rows on a greenhouse bench. The upper surfaces of the heart-shaped leaves of the plants in each alternate row were then sprayed, at 3:00 P.M. on a clear day, with 1 per cent naphthalene acetic acid emulsion. Leaves of plants in the remaining rows were sprayed on the upper surfaces with an emulsion of pure lanolin, to serve as controls. Initial samples were collected at random from both sprayed and control plants immediately after treatment. Samples were collected during the following night at 11:00 P.M.; and at 9:00 A.M., and again at 9:00 P.M.—following a dark cloudy day. Epinasty occurred within 90 minutes after treatment but otherwise there were no obvious differences between sprayed and control plants.

TABLE 1  
SUGAR, STARCH, AND DEXTRIN CONTENT OF SPRAYED AND CONTROL LEAVES. SAMPLING BEGAN AT END OF A CLEAR DAY, AND CONTINUED DURING THE NIGHT AND FOLLOWING DAY, WHICH WAS DARK AND CLOUDY. FIGURES REPRESENT AVERAGE OF DUPLICATE DETERMINATIONS OF PERCENTAGE OF SUGAR, STARCH, AND DEXTRIN, EXPRESSED ON DRY WEIGHT BASIS

TIME OF SAMPLING	HOURS AFTER SPRAYING	PERCENTAGE			
		SUGAR		STARCH AND DEXTRIN	
		CONTROL	TREATED	CONTROL	TREATED
3:00 P.M. ....	0*	2.4	2.4	3.8	3.8
11:00 P.M. ....	8	2.1	2.8	2.8	2.7
9:00 A.M. ....	18	1.6	3.2	1.5	0.5
9:00 P.M. ....	30	1.0	1.6	1.8	0.0

\* Sampled at time of treatment.

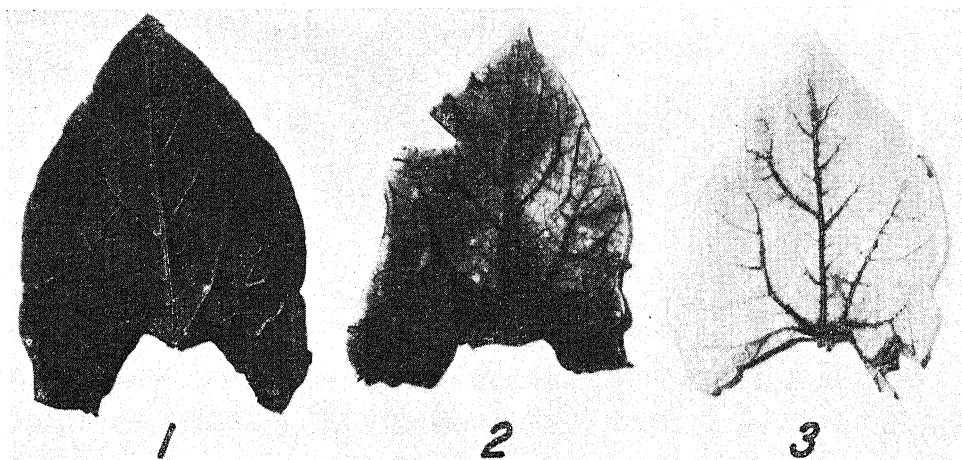
Table 1 shows that the starch and dextrin content of sprayed leaves decreased until finally the leaves contained an immeasurable amount of these substances, while only approximately one-half the starch and dextrin was hydrolyzed in control leaves during the same interval of time. The difference in starch and dextrin content of sprayed and control leaves was not noticeable until at least 8 hours after treatment.

Iodometric tests made at frequent intervals during the experiment likewise showed marked decrease in starch content of sprayed leaves after approximately the fifteenth hour following treatment.

The sugar content of sprayed leaves increased appreciably above that of controls following application of the acid, but later decreased rapidly as starch digestion continued, and finally reached approximately the same percentage concentration as that of controls at the time the experiment was concluded.



A second experiment was performed in which the digestion of starch in sprayed leaves was studied during a prolonged period of darkness, in order to eliminate any effects due to photosynthesis. The plants used in this instance were older than those of the previous experiment and the starch content of the heart-shaped leaves at the beginning of the experiment was greater. To facilitate handling, the plants were placed on trucks so that they could be easily transferred to a darkroom immediately following the spraying. The upper surfaces of the primary leaves of half the plants were sprayed as previously with 1 per cent naphthalene acetic acid emulsion and the leaves of the remaining plants were sprayed with emulsion of pure lanolin. The plants were placed in the dark immediately after



FIGS. 1-3.—Starch content of representative leaves as shown by iodine test: fig. 1, at time of treatment; fig. 2, after 19 hours of darkness following spraying with pure lanolin emulsion; fig. 3, after 19 hours of darkness following spraying with 1 per cent naphthalene acetic acid-lanolin emulsion.

treatment and initial samples for chemical analysis were collected at random. Subsequent samples were collected as previously described at intervals during the following 48-hour period. The temperature to which the plants were exposed during the experiment varied between 70° and 75° F.

The percentage of starch and dextrin in both sprayed and control leaves increased slightly for several hours after the plants were removed from natural daylight of high intensity and placed in darkness (table 2; fig. 4). Subsequent to the initial increase, the starch and dextrin contents of both sprayed and control leaves decreased at approximately the same rate until the fourteenth hour, after which time starch digestion progressed at a greater rate in sprayed than in control leaves. Similar results were shown by means of the iodine test, which was made repeatedly during the experiment (figs. 1-3).

As in the previous experiment, the percentage of sugar in leaves to which the acid emulsion was applied increased appreciably several hours after treatment, but remained at a relatively high level for a period of only a few hours, then decreased to approximately the same percentage as that of controls.

These data corroborate those of the previous experiment, in that the use of 1 per cent naphthalene acetic acid emulsion accelerated the rate of starch digestion in the leaves, and the increased rate of hydrolysis was associated with a relatively high sugar content that persisted until most of the starch and dextrin was digested.

TABLE 2

SUGAR, STARCH, AND DEXTRIN CONTENT OF SPRAYED AND CONTROL LEAVES. PLANTS CONTAINING RELATIVELY LARGE AMOUNTS OF SUGAR, STARCH, AND DEXTRIN WERE TREATED, IMMEDIATELY PLACED IN THE DARK, AND SAMPLES COLLECTED. FIGURES REPRESENT AVERAGE OF DUPLICATE DETERMINATIONS OF PERCENTAGE OF SUGAR, STARCH, AND DEXTRIN EXPRESSED ON DRY WEIGHT BASIS

HOURS AFTER SPRAYING	PERCENTAGE			
	SUGAR		STARCH AND DEXTRIN	
	CONTROL	TREATED	CONTROL	TREATED
0*.....	2.1	2.1	9.7	9.7
3.....	1.6	1.6	10.6	10.3
6.....	1.5	1.5	10.3	9.5
10.....	1.3	2.3	9.4	9.2
14.....	1.4	2.5	9.3	9.5
20.....	1.1	2.6	8.5	6.2
30.....	1.1	1.6	6.8	3.8
48.....	0.9	0.8	4.3	2.4

\* Sampled at time of treatment.

Data from additional experiments, in which indoleacetic acid and phenylacetic acid were substituted individually for naphthalene acetic acid, showed that these substances also accelerated the rate of starch hydrolysis. In these experiments the effect of these substances on the starch content of leaves was demonstrated by means of iodine tests.

A third experiment was conducted to study the effect of sprayed naphthalene acetic acid emulsion on the accumulation of sugars, starch, and dextrin in the heart-shaped leaves of beans. After the terminal buds of relatively young plants had been removed at the second node, the plants were placed in darkness until the leaves were depleted of starch as shown by iodine tests. The leaves of half the plants were then sprayed on the upper surfaces with 1 per cent naphthalene

acetic acid emulsion as previously described, and the remaining half with lanolin emulsion. The sprayed and control plants were immediately transferred from

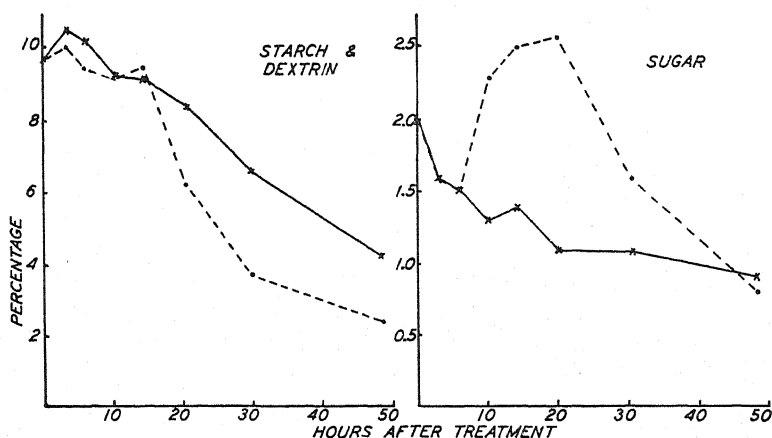


FIG. 4.—Percentage of sugar, starch, and dextrin in primary leaves of bean plants sprayed with 1 per cent naphthalene acetic acid emulsion and kept in the dark for 48 hours (broken lines), as compared with comparable leaves sprayed with pure emulsion (solid lines). Percentage calculated on dry weight basis.

TABLE 3

SUGAR, STARCH, AND DEXTRIN CONTENT OF SPRAYED AND CONTROL LEAVES. PLANTS PLACED IN DARK UNTIL SUGAR AND STARCH CONTENT WAS DEPLETED, THEN EXPOSED TO NATURAL DAYLIGHT. FIGURES REPRESENT AVERAGE OF DUPLICATE DETERMINATIONS OF PERCENTAGE OF SUGAR, STARCH, AND DEXTRIN EXPRESSED ON DRY WEIGHT BASIS

TIME OF SAMPLING	HOURS AFTER SPRAYING	PERCENTAGE			
		SUGAR		STARCH AND DEXTRIN	
		CONTROL	TREATED	CONTROL	TREATED
7:00 A.M. . . . .	0*	0.34	0.34	0.0	0.0
8:00 A.M. . . . .	1	0.9	0.9	0.0	0.0
9:00 A.M. . . . .	2	1.7	1.6	0.0	0.0
10:00 A.M. . . . .	3	3.2	2.5	Trace	Trace
11:00 A.M. . . . .	4	4.2	2.9	2.6	1.6
12 NOON. . . . .	5	4.9	3.0	2.6	2.3
1:00 P.M. . . . .	6	5.9	3.4	6.1	4.4
2:00 P.M. . . . .	7	6.3	3.5	7.6	4.6
3:00 P.M. . . . .	8	5.5	3.6	8.0	5.9
5:00 P.M. . . . .	10	5.7	3.8	9.3	6.4

\* Sampled at time of treatment.

the darkroom to the greenhouse, where they were arranged in alternate rows. They were illuminated by means of natural daylight, and duplicate samples were

collected from sprayed and control leaves at the end of each hour for a period of 10 hours. The light intensity increased from 750 foot-candles at 7:00 A.M. to 2000-3000 during the middle of the day, then decreased to approximately 500 foot-candles at 5:00 P.M. On several occasions during the day the sun shone brightly and the intensity reached a maximum of approximately 10,000 foot-candles during short intervals. The temperature was 75° F. at the beginning of the experiment and increased during the morning, reaching a maximum of 90°-92° during the interval between 1:00 and 5:00 P.M.

The rate of sugar accumulation was approximately equal in sprayed and control leaves during the 2 hours immediately following treatment, but after this interval control leaves accumulated more sugars than did the sprayed leaves (table 3). The accumulation of starch was likewise less in sprayed than in control leaves.

### Discussion

When certain concentrations of mixtures of naphthalene acetic acid and lanolin are applied to the stems of actively growing bean plants, many of the cells composing certain regions of the stem—such as the endodermis, phloem, cambium, rays, and pith—manifest greatly increased meristematic activity (1, 2, 3). This is true both for embryonic cells and for those which show advanced stages of maturation in differentiation, wall thickening, vacuolization, and food storage. If, as in the latter type of cells, they contain starch, one of the first readily detectable changes is the disappearance of the starch grains, accompanied by a general increase in the density of the cytoplasm, sometimes an enlargement of the entire cell, and very often subsequent cell division. There is in effect a restoration of the embryonic condition in many of the cells which have already differentiated. It is probable that the reserve carbohydrates, dextrin, starch, and hemicellulose are hydrolyzed to less complex forms of carbohydrate in cells which respond to treatment with naphthalene acetic acid, and that such forms may be more rapidly and more readily metabolized.

From the data presented it is evident that naphthalene acetic acid increased the rate of starch digestion in the leaves of beans to which it was applied. The results indicate that the acid may have affected diastatic activity indirectly, as 10-15 hours elapsed following treatment before an appreciable difference was noted between the starch and dextrin contents of the sprayed and control leaves. Associated with more rapid digestion of starch was a marked increase in the sugar content of the sprayed leaves. This increase was observed in sprayed leaves kept in darkness, and when the digestion of starch neared completion their sugar content decreased and finally approached that of control leaves. It is therefore probable that this temporary accumulation of sugars in sprayed leaves was, partially at least, caused by the increased rate at which complex forms of carbohydrates were hydrolyzed owing to application of the naphthalene acetic acid.

It is also probable that the application of 1 per cent naphthalene acetic acid retarded the accumulation of carbohydrate in leaves, as both the sugar and starch contents of sprayed leaves were less, during a period of illumination, than that of controls. Further studies may determine whether this sugar content of sprayed leaves resulted from increased respiration, transport, or lower rate of photosynthesis.

### Summary

1. A 1 per cent naphthalene acetic acid-lanolin and water emulsion was sprayed on the upper surfaces of attached bean leaves. The digestion of starch and accumulation of sugars during a period of darkness or low light intensity and the rate of accumulation of sugar and starch during a period of illumination were studied by means of quantitative chemical determinations made at intervals following treatment.

2. The percentage of starch and dextrin in leaves sprayed with naphthalene acetic acid emulsion and placed in darkness decreased more rapidly than did that of control leaves. Noticeable differences, however, were not apparent for several hours after treatment. The percentage of sugar in leaves sprayed with the acid emulsion and placed in darkness increased during that period following treatment when starch digestion was most rapid, but later decreased as the starch reserve was depleted, finally reaching a value approximately equal to that of untreated leaves.

3. Leaves that had been depleted of sugars, starch, and dextrin by being kept in the dark and subsequently sprayed with naphthalene acetic acid emulsion and then placed in natural daylight accumulated less sugar, starch, and dextrin during the period of illumination than did comparable untreated leaves.

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# EVOLUTIONARY SIGNIFICANCE OF RING POROSITY IN WOODY ANGIOSPERMS

S. G. GILBERT

(WITH TWELVE FIGURES)

## Introduction

With the increasing use of wood anatomy in phylogenetic investigations, it is important to check carefully the principal lines of specialization in xylem structure. One of the best known features of gross anatomy is the arrangement of the elements, especially the large spring vessels in deciduous trees. It is generally considered that ring porosity represents a high degree of such specialization; but there has been disagreement as to whether such a structural modification has any marked evolutionary significance or represents merely an ecological adaptation. The present study attempted to settle this question and also to determine the direction and nature of such an evolutionary change with respect to angiosperm origin.

Recent work by HUBER (15), PRIESTLEY (28), and LODIEWICK (26) on the conduction of water and the nature of cambial activity indicates that this structural difference has definite physiological implications. Because of this it is generally considered that ring porosity represents a specialization from the diffuse porous condition, yet there appears to have been no serious morphological study of this viewpoint. HUBER believes—on purely physiological evidence—that the ring porous condition is a phylogenetically advanced feature allowing rapid conduction of water in the early spring followed by formation of the more drought-resistant summer wood.

The only other discussions of the evolutionary origin of the two types that were found in the literature are by GROOM (14), FROST (9), and BAILEY (2). The first of these deals with *Quercus* alone. GROOM points out that three main courses of evolution are possible in deriving the annual ring of the oaks. These he gives as follows:

- “1. Regressive from a typical ring with marked pore-zone.
2. Divergent from a ring with a feebly marked pore-zone.
3. Progressive from a ring with no indication of a pore-zone.”

GROOM does not commit himself as to which of these possibilities represents the actual evolutionary course. The fact that he worked with only the mature wood may have led to his hesitancy, for this genus exhibits almost all the possible types of vessel arrangement.

On the other hand, FROST states: "Ring porous woods tend to have larger vessels than diffuse porous woods. There is a high correlation between the diffuse porous condition and the presence of scalariform perforations. As one might expect, the distribution of primitive vessels follows the distribution of tracheids (that is, diffuse or more or less evenly spaced throughout the xylem)" (9).

No data were given by FROST to support this conclusion, and a check made on the tables of BAILEY and TUPPER (4)—the basis of FROST's work—showed no such correlation. This reconsideration is discussed later in this paper. The general conclusion of FROST is still tenable, however, that the primitive type of vessel arrangement was diffuse.

BAILEY (2) also states that the ring porous condition is highly specialized. "The typical ring porous condition arises apparently when plants which have undergone characteristic structural modifications in certain tropical or subtropical environments are subjected to cold winters or to alternating very dry and wet seasons. In other words this is closely associated with the acquisition of a pronounced resting period and the commencement of the deciduous habit. It is significant that certain species of a widely dispersed genus may be ring porous and others diffuse porous, depending upon their phytogeographical distribution." He regards this as evidence of parallel lines of specialization.

These generalizations seem to be plausible, but little concrete evidence was advanced to support them. Pure speculation can also make a possible case for the opposite point of view—that the diffuse porous type is a reduction from the ring condition. The Amentiferae are considered primitive by many systematists; this group has a number of ring porous species. The Gnetales have been postulated as close to the point of angiosperm origin; the most generally primitive member of this family (*Ephedra*) is ring porous (fig. 1).

As SINNOTT and BAILEY (38) have shown, the tree form is to be regarded as the primitive type for angiosperms. If this is so, then the question of the structural pattern of the wood becomes important in any attempt at a reconstruction of the ancestral race. If neither is primitive, one is forced to assume a marked polyphyletic origin of the present groups. While this possibility must be logically allowed, it is contrary to the trend of modern concepts of morphology.

In the present investigation two methods of attack are employed, one qualitative and the other more quantitative in nature. The first is based on the principles developed by JEFFREY and his students. As they have been extensively described in the literature (16, 17, 3, 35), a brief outline will suffice here. The theory of recapitulation is used as a basis for postulating that there can exist "conservative" regions in the plant body that will retain more primitive features of organization than will the others. Moreover, under the stimulus of certain types of injury reversion may occur to the phylogenetically original morphological feature. Thus

in attacking the present problem, the anatomy of the first annual ring, node, root, and reproductive axis has been investigated in addition to the study of seedling structure. Some attention has also been given to the effect of traumatism upon ring porosity.

Within the last 20 years there has been developed another promising method of studying evolutionary trends in specializations of wood anatomy. The foundations of this were principally laid by the work of BAILEY and TUPPER (4). FROST (9, 10) has given a discussion of the theory and application of this method.

The technique depends on the previous establishment of an undoubtedly primitive anatomical feature. This is then associated with the feature to be investigated to determine the extent and direction of the correlation between the occurrence of both features in the various species. A high positive correlation would indicate that the feature studied is relatively primitive. If a similar characteristic shows a more negative correlation with the established primitive feature, it would be considered more advanced and the line of specialization could then be read from the first to the second characteristic.

FROST applied this method in a study of the development of the vessel. He used as his original primitive characteristic the length of the tracheary elements. BAILEY and TUPPER had already shown that the most advanced element was the shortest one. FROST associated the length of the vessel with the type of perforation and the end wall. He found a high degree of correlation between the primitive conditions of length and scalariform perforation. From data such as these he concluded that the vessel was derived from the scalariform tracheid and that the wood of the homoxylous angiosperms (*Trochodendron*, *Drimys*, etc.) "is unquestionably very primitive."

These same methods were used by KRIBS (23, 24) in studying the salient lines of specialization of the wood rays and wood parenchyma of dicotyledons. He used as his basis the vessel types of FROST. According to KRIBS' conclusions the specialized types are the homogeneous ray and the vasicentric abundant distribution of wood parenchyma.

It is generally agreed (16, 11) that the fibrous wood elements have been derived from tracheids by a reduction of the bordered pit to the simpler nonbordered condition. A wood with its fibers having definite borders on the pits is regarded as showing a more primitive condition with respect to this feature.

In this paper the presence of the ring porous vessel arrangement was associated with each of these advanced wood structures, and the degree of correlation determined. Unfortunately the extensive material necessary for a really statistically sound investigation was not obtainable. The data, however, were already listed in a number of reference works on wood anatomy (29, 30, 44, 39, 12, 27, 33, 40). The paper of BAILEY and TUPPER (4) was of particular value in this connection. In



view of these extensive data on the wood anatomy of the various species, it was decided that good indication of the correlation trends could be obtained by a thorough search through the literature and the statistical use of the accumulated data.

One important feature of the present application of this method is to be noted. It is well known to students of wood anatomy that the ring porous type is almost anomalous in consideration of the overwhelming preponderance of diffuse porous woods in the flora of the world. As will be emphasized later, occurrence of the ring condition is restricted to a geographical region—the North Temperate zone. In consideration of the effect of environment on plant distribution and evolution, it is regarded as unsound to attempt to correlate on a world-wide basis the various lines of specialization with the development of the ring porous condition. Such studies must be made only with plants of the North Temperate zone. The necessity for such restriction is obvious since it is manifestly impossible for specializations in wood structure occurring in the tropics to have any relationship to ring porosity, when that type does not and cannot exist under the climatic conditions characteristic of tropical regions. The presence of a diffuse arrangement in a tropical species is no criterion of a primitive wood.

There is no indication that FROST (9) has considered this qualification in the use of the data. This may explain the inaccuracy of his statement regarding “the high correlation between the diffuse arrangement of vessels and the scalariform condition of the end wall.”

### Material and methods

The material consisted of the wood of different organs of various ages and locations from about sixty different species, representing eleven families.<sup>1</sup> The writer is responsible for all identifications except for the material from the Sonderegger and United States Soil Conservation nurseries. Almost all the collecting was done in the neighborhood of New Brunswick, with the exception of some material from South Jersey and that of *Quercus virginiana* which was obtained from Florida through the kindness of Dr. M. A. CHRYSLER. I am indebted to Dr. FORREST SHREVE for the specimens of *Ephedra trifurca* from Arizona.

Sections were made of the woody cylinder of the different organs of plants of various ages. In general, vigorous, well grown material was selected. In most cases only cross sections were made, and therefore the term vessel as used in this paper strictly includes any tracheary element of diameter greater than about 0.05 mm. No serious error is introduced by this convention, since the diagnostic vessels of a ring porous type are much larger than this (at least five times), and tracheids are generally smaller.

<sup>1</sup> Details of material and results will be found in table 1 of the thesis submitted in partial fulfillment of the Master of Science degree, Rutgers University. 1938.

### Experimental work

#### A. EVIDENCE FROM COMPARATIVE ANATOMY

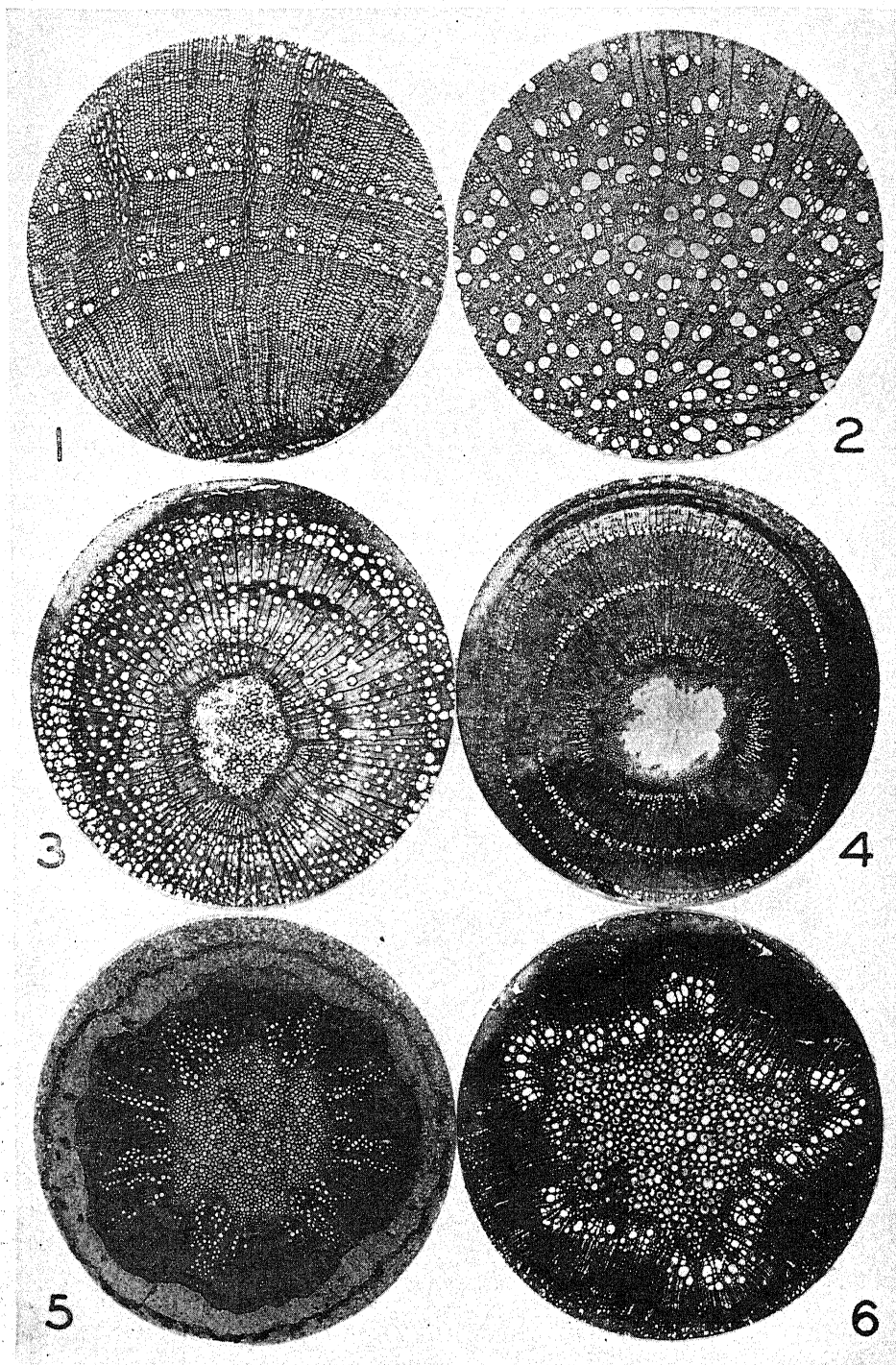
1. ROOT.—Roots of all the investigated genera were examined and found to be diffuse (fig. 2). RIEDL (31) found this condition to be so constant that the use of this feature was not possible in the construction of a systematic key for the identification of root wood. Wounded root wood of *Ulmus americana* showed only the diffuse condition in all parts adjacent to and at the point of injury. This is only negative evidence, of course, and is not of theoretical importance since reversions do not necessarily accompany all cases of injury.

2. FIRST ANNUAL RING AND NODE.—All diffuse porous species examined showed no essential difference in vessel distribution in the first annual ring as compared with the adult wood. In sections just below the node, however, there was a tendency toward grouping of the larger vessels in the metaxylem and early secondary xylem associated with the "leaf supply" to the leaves immediately above. This was especially evident in *Acer platanoides* and *Fagus americana* (figs. 4, 7, 10). As this was also seen in sections of many of the ring porous types and seemed to be a special condition associated with the leaves, no special recapitulatory significance was attached to it. But as the metaxylem and early secondary xylem of the leaf trace are continued basipetally in the progressively older portions of the annual ring, it is evident that continuous production of leaves throughout most of the annual increment of growth develops a diffuse condition of the vessels. Likewise any reduction of leaf production or restriction to an early spring phase with continued wood formation in the summer will lead to a markedly ring type.

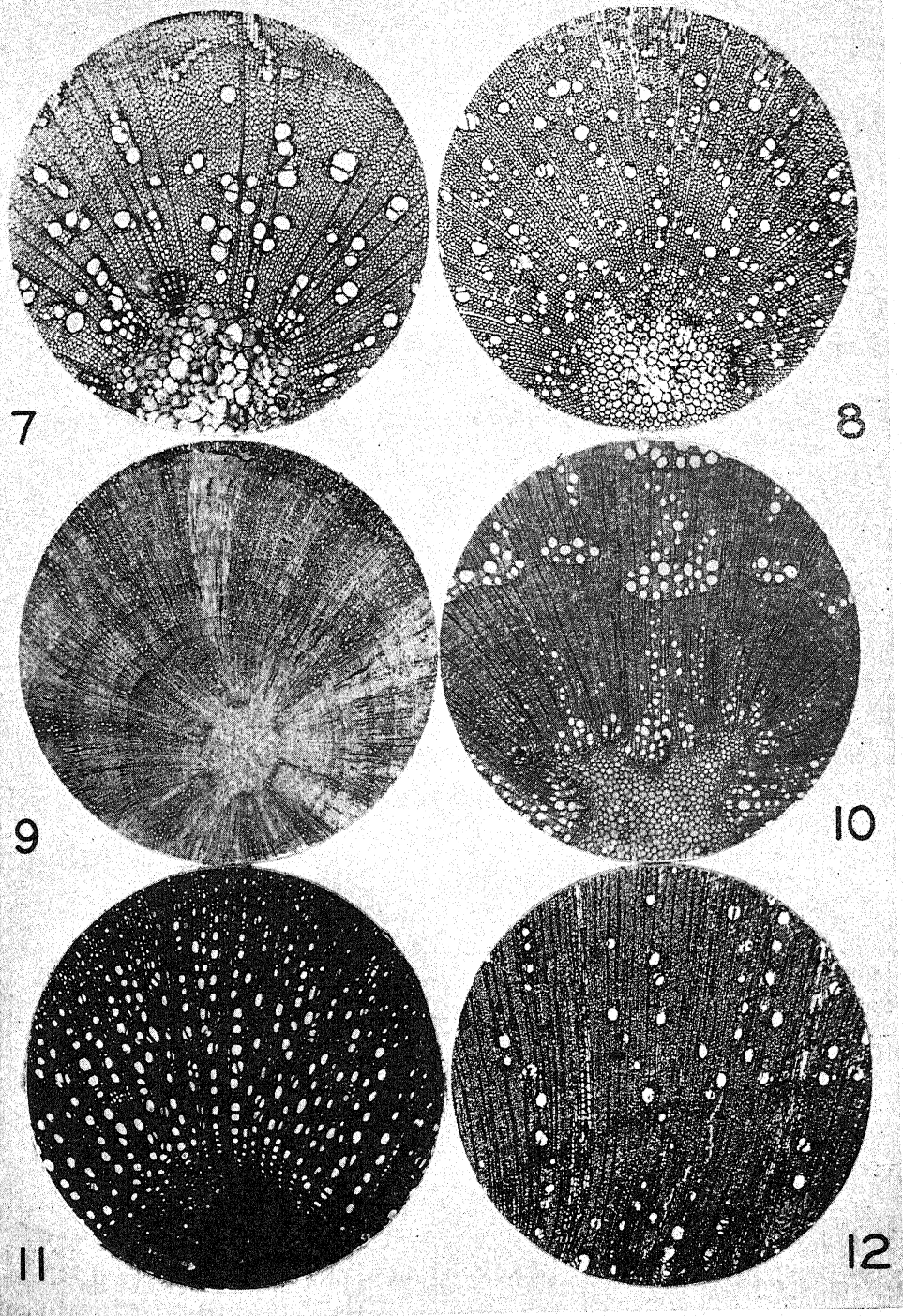
In almost all genera which are normally ring porous in the adult wood, the first year's growth was diffuse. In some instances this condition persisted through a number of subsequent annual rings before the adult condition was established (figs. 3, 9). In all probability this recapitulatory rate can be accelerated or made slower according to the environmental conditions present.

In some species of *Quercus* this tendency was poorly evident or could be considered as on the borderline. Examples of this are *Q. imbricata*, *Q. marilandica*, *Q. phellos*, and *Q. prinoides*. *Q. prinus*, on the other hand, gives evidence of a shorter recapitulation of this feature, being almost characteristically ring porous in the first annual ring of a twig from an adult tree. In these cases, however, the concave pith "depression" showed markedly greater diffusion of pores than the xylem opposite the pith arms. At the node the leaf gap, of course, showed only parenchyma.

From observation of serial sections through two successive nodes and study of young twigs stripped of their bark, it was evident that the fluted appearance of the twig was associated with the phyllotaxy.



FIGS. 1-6.—Fig. 1, *Ephedra trifurca*, branch showing ring porous arrangement. Fig. 2, *Ulmus americana*, normal root. Fig. 3, *Morus* sp., twig showing slow recapitulation. Fig. 4, *Ailanthus glandulosa*, young twig, rapid recapitulation. Fig. 5, *Quercus borealis*, seedling above hypocotyl. Fig. 6, *Q. alba*, seedling upper stem showing ring porosity.



FIGS. 7-12.—Fig. 7, *Robinia pseudo acacia*, hypocotyl near node. Fig. 8, *Celtis occidentalis*, vigorous seedling stem. Fig. 9, *Quercus ilicifolia*, seedling stem 10 years old, diffuse porous. Fig. 10, same, adult branch ring porous. Fig. 11, *Castanea dentata*, peduncle, diffuse porous. Fig. 12, *Quercus (velutina?)*, portion of traumatic tissue magnified to show diffuse arrangement of pores.

JEFFREY (16) calls the fluted appearance of the oak stem a "depression or retardation of growth," yet there is evidently a different set of factors at work here. The "depressed" segment is of exactly the same width as the rest of the annual ring (fig. 10). If anything, there is acceleration of growth, for in the latter rings of a branch the fluted appearance is lost, indicating that the growth rates must be slightly faster to correct for the earlier fluting. This effect is clearly the result of the type of cambial activity and primary meristematic growth associated with formation of the leaves. PRIESTLEY (28) has shown that the direction of xylem differentiation is basipetal. Thus in the formation of the vascular cylinder the earliest differentiation will be in that portion which will later be the leaf supply. Initiation of the rest of the stele will be associated with the leaves of the successively higher nodes. Thus retardation of both time and position of origin will be produced, but no differences in absolute growth rate will be evident.

The influence of phyllotaxy on the vascular anlage can be seen clearly in the figures of LANGDON (25). The vascular supply to the leaf was found by her to be formed before the leaves themselves were well differentiated in the seedling buds of *Quercus*.

3. SEEDLING ANATOMY.—Seedlings of eighteen species of ring porous dicotyledons were examined. In all cases the plants were at least one year old, and of good vigor compared with others of the same lot. Eleven species were from the nurseries of the Soil Conservation Service at New Brunswick and were selected as "vigorous." Poorly developed specimens were also examined to check the findings, but no significant differences were obtained. In the case of *Ulmus americana*, over twenty seedlings of varied growth status were compared. The results obtained were as follows:

- (a) All seedlings showed hypocotyls that were diffuse porous (fig. 7).
- (b) A similar condition was found at the cotyledonary node.
- (c) All first year seedling stems were likewise diffuse (figs. 5, 8, 9), with the exception of those examined of the genus *Quercus*. These showed a definite tendency toward the ring porous condition near the apex of the stem (fig. 6), although half way below this the condition was markedly diffuse (fig. 5).
- (d) The two-year-old seedling of *Fraxinus americana* of exceptional vigor was likewise diffuse the first year and ring the second. In *F. pennsylvanica*, a normal seedling of one year's growth showed the typical diffuse condition even at the apex of the stem.

4. PEDUNCLE.—In consideration of the well known conservatism of the reproductive organs, an examination was made of the floral axis of those plants characterized by the formation of a considerable cylinder of secondary wood. Members of the genus *Quercus* are particularly favorable for this purpose; especially so are the members of its subgenus *Erythrobalanus*, which retain their acorns for two



years, thus forming two annual rings in the floral axis. Herbarium material was used to insure identification of the species. Other genera used are noted below.

The three species of *Lepidobalanus* showed only a diffuse annual growth. This was especially marked in the large peduncle of *Prinus* but was also clear in *alba* and *bicolor*. All the species of *Erythrobalanus* were diffuse porous in both annual rings except *phellos*, which had a tendency toward ring porosity near the main axis. The region near the acorn base was decidedly diffuse porous in both rings. The peduncle of *Castanea dentata* was markedly diffuse porous (fig. 11). The floral axis of *Ailanthus glandulosa* was slightly ring porous near the abscission region and diffuse near the inflorescence.

It is evident that the data from the floral axis agree with the previous evidence in establishing the primitive nature of the diffuse porous condition.

5. WOUNDED STEMS.—Close examination of a number of cases of severe wounding in young oak branches showed definite signs of reversion to the diffuse condition as a result of the hypertrophy produced. This stimulating effect was especially marked in the sector formed directly from the injured portion and in the wound cap proper. Figure 12 shows the condition of the stem in the region adjacent to the wound.

The observed effects were an increase in the diameter of the summer vessels and reduction in size of the spring pores that were formed after wounding. A plausible explanation of this result may lie in the new growth situations present in the wounded region. The spring growth may have been retarded and the transition to the period of summer growth made less sudden by the effects of wounding followed by hypertrophy. The question awaits study of the physiological factors involved.<sup>2</sup> Whatever these factors may be, it is evident that in this case there is definite reversion to the ancestral type of vessel arrangement. It may be possible that the conditions of hypertrophy are analogous to the more favorable climatic conditions of earlier geological history.

As the physiological agents are so poorly understood and so important in this type of phylogenetic investigation, no great reliance is placed on this evidence. It is of significance only in relation to the more reliable data that have been obtained. The results are recorded principally to show that these data do not conflict with the other findings. It is to be noted, however, that BAILEY (1) does not record such traumatic effects in his study of wounded oak stems.

## B. CORRELATION STUDIES

A study of the correlation between the presence of ring porosity and certain other advanced features of wood anatomy has been made. The results are given in table 1. The families considered are listed in tables 10 and 11 of RECORD (30).

<sup>2</sup> A recent paper (21) on day length is of interest in this connection.

The results of this investigation show in general such high correlation that they may be regarded as conclusive, even though the data are admittedly incomplete with regard to exotic genera.

There is perfect correlation between the ring porous condition and the most advanced type of vessel wall perforation, in so far as genera are concerned. There is probably a negative correlation in the case of the indigenous diffuse porous genera. The correlation in regard to the families with ring porous representatives is almost as perfect, but no such correlation can be demonstrated for the families whose indigenous representatives are exclusively diffuse porous. In view of

TABLE 1

STATISTICAL STUDY OF SALIENT LINES OF STRUCTURAL SPECIALIZATION AMONG  
RING POROUS AND DIFFUSE POROUS GENERA AND FAMILIES

CLASS	VESSEL PERFORATION		PITTING ON WOOD FIBER		DOMINANT RAY TYPE		WOOD PARENCHYMA DISTRIBUTION	
	SIMPLE	SCALARI-FORM	SIMPLE	BORDERED	HETERO-GENEOUS	HOMO-GENEOUS	PARATRA-CHEAL	OTHER TYPES
Indigenous ring porous genera†.....	53	0	27	6	18	14	52	1
Indigenous diffuse porous genera†.....	9	18	3	27	23	12	2	18
Indigenous ring porous families†.....	20	4*	22	3	16	4	13	1
Indigenous diffuse porous families†.....	24 (Record)	20	9	21	15	3	0	25
	23 (Bailey)	23						
All diffuse porous families in entire world†.....	59	37	75	80	.....	.....	.....	.....

\* Mostly simple perforations.

† Exclusively diffuse type.

‡ Some ring porous members present.

Frost's statement (9, p. 74), there is special significance in the world-wide data on families whose tree members are exclusively diffuse porous. If anything, the figures show a weak correlation between diffuse porosity and advanced vessel type.

Two of the other features associated with ring porosity in this study agree with the preceding findings in regard to the advanced nature of the ring porous type. These are the nature of the pitting of the wood fiber wall and the distribution of wood parenchyma. The other, the type of ray, shows nothing of phylogenetic significance in an attempt to correlate either of the main types with the vessel pattern. It is also to be noted that the heterogeneous ray is the dominant type in temperate North America.<sup>3</sup>

<sup>3</sup> The numerous genera of the Leguminosae are of the homogeneous ray type. On the basis of the families, the heterogeneous ray is the dominant type in the ring porous families (table 1).

### Discussion

Ring porosity is restricted to a definite geological region, that of the North Temperate zone. Members of the same genus, and even supposedly the same species, may be ring porous in one region (for example, North America) while farther south they show the diffuse condition exclusively. The Leguminosae, for example, have about sixteen genera of trees in North America and all are ring porous. There are more than 125 genera in tropical South America and all are diffuse porous. The genera *Quercus*, *Ulmus*, *Celtis*, and *Trema* also show this feature clearly (29, 44). The Southern Hemisphere, even in the temperate regions, has no listed ring porous trees.

The evidence from the fossil record, incomplete as it is, shows certain pertinent facts regarding the possible origin of the ring porous type. JEFFREY (16) has shown that the late Carboniferous represents the beginning of the annual ring, with the geographical origin of this feature in the boreal regions.

The climatic conditions associated with the formation of the annual growth habit cannot be restricted to the Northern Hemisphere, for SAHNI (34) and BARNARD (5) have described fossil woods with annual rings from the early Tertiary of the southeastern regions of the world. GOTHAN (13) found two species of *Laurinoxylon* and certain coniferous woods from the Upper Cretaceous and Tertiary of the Antarctic that show definite rings. The earliest known woods of a definite angiosperm nature show no signs of a ring porous arrangement, even though well developed vessels are present (37, 41, 42).

While the fossil wood of the early Tertiary is mainly diffuse porous, the Miocene is characterized by the occurrence of the transitional type. FELIX (7), KNOWLTON (20), and JEFFREY and CHRYSLER (18) have described such types. The succeeding epochs show the development of all the present day genera of trees, although some of them had a more extended range in the interglacial periods (6).

There is abundant evidence to indicate that the ring porous type of vessel arrangement represents an evolutionary advance from the diffuse distribution. This conclusion is based on the agreement of the results of investigations using such different methods of attack as that of the comparative morphology of JEFFREY's school and the statistical approach of BAILEY and his students. Thus it has been shown that the evidence from the seedling, first annual ring, root, and the reproductive axis points definitely to the primitive nature of the diffuse porous type. The evidence from the traumatic phenomena is to be regarded as inconclusive in itself, although definite indications of reversion to the primitive arrangement are to be found in severely wounded stems of *Quercus (velutina?)*.

A high degree of correlation has been shown to exist between ring porosity and



the presence of structural features whose advanced nature has been universally conceded. These are the simple perforation of the end wall of the vessel element, the paratracheal distribution of wood parenchyma, and the presence of only simple pits on the wood fibers. This fact is strikingly evident in the lower families having ring porous members. In such cases the diffuse porous genera and species of the North Temperate zone tend to have the primitive features, while the ring porous ones are almost entirely specialized.

In the Juglandaceae, *Carya*, which is the characteristically ring porous genus, has pitting on the wood fibers with such faint borders that SOLEREDER considers this a genus with the simple pit feature and distinguishes it from the other genera on this basis. *Platycarya*, a monotypic genus of Asia, is of a less marked ring porous nature, and has definite bordered pits on its wood fibers (22). The other genera are diffuse porous and show the bordered condition. Only simple perforations are found in the first two genera, and also in the widespread genus *Juglans*, which shows a tendency toward ring porosity in two species (*J. cinerea* and *J. nigra*). The other two genera, *Engelhardtia* and *Alfaroa*, are of restricted range, diffuse porous, and with some to many vessel elements with scalariform perforations.

In considering the phylogenetic distribution of the ring porous feature, one is impressed with the fact, suggested by BAILEY (2), that so many important families of different positions in the ENGLER-PRANTL system of classification have ring porous representatives. This would seem to indicate either a polyphyletic origin of this feature or an early appearance before there was marked diversification of the primitive angiosperms. The finding of wood like *Laurinoxylon* from the beginning of the Tertiary lends support to the latter possibility.

WIELAND (43) has called attention to the hypothetical derivation of the angiosperm wood from the Cycadeoids of the Cretaceous and Jurassic through a homoxylous type as an intermediary. Whatever may be the status of the Cycadeoids in this question, the vessel-less angiosperms have assumed increasing importance in tracing angiosperm origin. The wood of *Pataloxylon scalariforme* (5) is especially interesting in that its sole conducting elements were large scalariform tracheids, while the other species *P. porosum* had both large tracheids and scalariform vessels. SAHNI (36) found a fossil wood (*Homoxylon rajmahalense*) with marked affinity to the living homoxylous Magnoliaceae (*Drimys*, *Tetracentron*, etc.). This fossil was "probably Jurassic." Similar woods have been described from the early Tertiary of Greenland and Germany (43). It should be noted, however, that JEFFREY and COLE (19) do not regard the vessel-less members of the Magnoliaceae as primitive, but as reduced. More recent anatomical work (9, 32) has tended to agree with the fossil evidence in regard to the primitive nature of the homoxylous wood.

The other Magnoliaceae are closely related to these genera and are likewise considered to be of early origin. This, however, does not preclude some cenogenetic evolution in successful genera like *Magnolia*. Through the courtesy of Dr. WILLIAM E. ROEVER, access was given to his abundant material of wood of eleven species of the Magnoliaceae. The only species showing any signs of a tendency toward ring porosity was *M. acuminata*. In a private communication, ROEVER stated that he had likewise noticed this tendency. This fact is especially interesting since earlier (32) he regarded the species as having in general the most advanced wood structure of those investigated.

Thus it is entirely within the realm of scientific possibility that the ring porous type may have developed as the result of a change in the climatic conditions of the late Cretaceous. An increasing aridity would first produce the more efficient *Fagoxylon* type of wood with its wide vessels. The development of a winter season followed by spring thaw and summer drought would produce marked seasonal inequality in the available moisture supply. This condition is that occurring in the present oak-hickory subclimax forest (8). The beech-maple climax is of a more mesophytic nature, however, and more closely akin to the supposed Cretaceous climate. This factor was probably at work in producing the abundant Miocene and Pliocene ring porous flora.

Those families which developed their taxonomic entity after this specialization of pore arrangement could have retained ring porosity as a genetical possibility, requiring only the proper habitat for its expression. Thus it is seen that those members of the higher families (Bignoniaceae, Papilionaceae, Scrophulariaceae, etc.) which have tree forms in the North Temperate zone are exclusively of the true ring porous type.

The diffuse porous members of the most advanced families are not therefore to be regarded necessarily as of more primitive origin than the ring porous members of the lower groups, or even of their own group. Ring porosity represents only one of the many possible lines of specialization that could be followed in developing a plant body that would be successful in a given environment. Conditions in the tropics are obviously not conducive to the formation of the ring arrangement, and this feature is to be regarded as of no phylogenetic significance in those regions. The temperate zones, however, afford an opportunity for the study of the development of this type of arrangement.

### Summary

1. The problem of the evolutionary significance of the ring porous type of vessel arrangement has been investigated. Two methods were employed, one based on that of JEFFREY and the other on the correlation method of BAILEY and his

students. Both methods gave identical results, indicating that they are both valid in this type of problem.

2. Data from the anatomy of the seedling, first annual ring, root, and reproductive axis show that diffuse porosity is the more primitive condition for trees which are ring porous in the adult stem.

3. A high degree of correlation has been shown to exist between ring porosity and the presence of structural features whose advanced nature has been generally conceded. These are the simple perforation of the vessel segment end wall, the paratracheal distribution of wood parenchyma, and the presence of only simple pits on the wood fibers. The ray type does not show this correlation. From these data it is concluded that ring porosity is an advanced feature.

4. The evidence indicates that this specialization took place early in angiosperm history and affords no proof for the existence of parallel lines of evolution. Ring porosity has probably developed as a response to the climatic conditions characteristic of the North Temperate zone. These conditions are now peculiar to a wide but delimited region of the world, which also represents the modern range of the specialized type. Any morphological considerations of this structural specialization are valid only within the limits of that range.

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# RESPONSE OF THE PEANUT PLANT TO INOCULATION WITH RHIZOBIA, WITH SPECIAL REFERENCE TO MORPHOLOGICAL DEVELOPMENT OF THE NODULES

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(WITH ELEVEN FIGURES)

## Introduction

Although nodules of the peanut, *Arachis hypogaea* L., were noted by POITEAU (27) as early as 1853, only occasional mention of the plant was made in nodulation studies until 1917, when BURRILL and HANSEN (6) placed it in the cowpea cross-inoculation group. Only recently has attention been given the plant in nitrogen-fixation studies. In the present investigation two phases of the rhizobium-leguminous plant complex have been studied: (a) the infective and effective abilities of various strains of rhizobia upon the growth of the plant, and (b) the histology and cytology of the nodules, the development of which apparently has not been heretofore described in detail.

## Greenhouse studies

Two commonly cultivated varieties, the Spanish and the Jumbo, were used in the tests. Plants of the former variety tend to grow erect, bear fruit around the base of a single stem, and can withstand considerable moisture; those of the latter are the trailing type and have greater xerophytic tendencies. Prior to planting, the shelled seeds were surface-sterilized in 1:1000 mercuric chloride for several minutes, washed in sterile water heated to 56°–60° C., rinsed in cold, sterile distilled water, and dried overnight at room temperature.

The inocula consisted of fifty-nine strains of rhizobia isolated from a variety of leguminous plants. The infective and effective abilities of these strains for other leguminous species have been noted previously (1).

The sterilized seeds were planted with the micropyle downward in sterile quartz sand. Ten-day-old cultures of the organisms in asparagus-potato mannitol broth were applied in the usual manner and the seeds covered with sterile sand. Germination, as evidenced by swelling of the seeds and their protrusion through the sand, usually occurred about the fourth day, although the plumule did not emerge until about the tenth day. By that time an appreciable root system had been formed. The growing period was 10–12 weeks, or until a considerable number of gynophores on each plant had penetrated the sand and were in the process of setting seed. The plants were watered as often as necessary with sterile distilled water and received Crone's solution weekly. The results of the greenhouse tests

have been based on a series of replicated experiments in which the strains of rhizobia were run in triplicate against both varieties. At the time of harvest the roots were examined in trays of water to facilitate detection of the different types of nodules.

The results are shown in table 1. The data obtained with the two varieties have not been tabulated separately, since the differences in types of nodules produced and the responses in growth were identical. Nodules usually made their appearance about the time the third set of leaves was forming, while their effect upon growth of the plant was not apparent until the fifth week.

TABLE 1

RESPONSE OF PEANUT PLANT TO INOCULATION BY VARIOUS STRAINS OF RHIZOBIA  
ISOLATED FROM TROPICAL LEGUMINOUS PLANTS

LUXURIANT GROWTH, DARK-GREEN FOLIAGE, MODERATELY LARGE NODULES ON CENTRAL ROOT SYSTEM	POOR STUNTED GROWTH, YELLOW TO YELLOW- ISH-GREEN FOLIAGE, INNUMERABLE SMALL NODULES THROUGHOUT ROOT SYSTEM
GROUP A: GOOD STRAINS	GROUP C: INEFFECTIVE STRAINS
Indigofera suffruticosa 7-4, 7-5	Phaseolus lathyroides 1-4, 1-5
Samanea saman 13-5, 13-7	Cassia mimosoides 5-2, 5-3
Arachis hypogaea 19-6, 19-7, 19-8	Andira inermis 20-1, 20-2
Desmodium barbatum 22-1, 22-2*	Vigna marina 27-1, 27-2
Stylosanthes guianensis 23-1, 23-3	Canavalia campylocarpa 32-6, 32-7
Erythrina indica 36-1, 36-4	Enterolobium cyclocarpum 34-7, 34-10
Albizia lebbek 39-1, 39-3	Lonchocarpus domingensis 74-1, 74-5
Alysicarpus vaginalis 77-3, 77-6	Piscidia erythrina 75-4
GROUP B: MODERATELY GOOD STRAINS	Derris microphylla 86-2, 86-3
Acacia koa 15-3, 15-4	Tephrosia candida 107-1, 107-3
Cassia chamaecrista 58-6	Hymenaea coubaril 100-1, 100-2
Piscidia erythrina 75-1	Inga laurina 108-1, 108-2
Parkia africana 89-1, 89-2	Pongamia pinnata 87-2, 87-4
Cytisus scoparius 2	Vigna sinensis 602, 603, 604
Glycine hispida 518	Glycine hispida 534, 500
Crotalaria spectabilis 9-5	Lupinus arboreus 1, 3, 7

\* Now strain 625 of the Wisconsin collection.

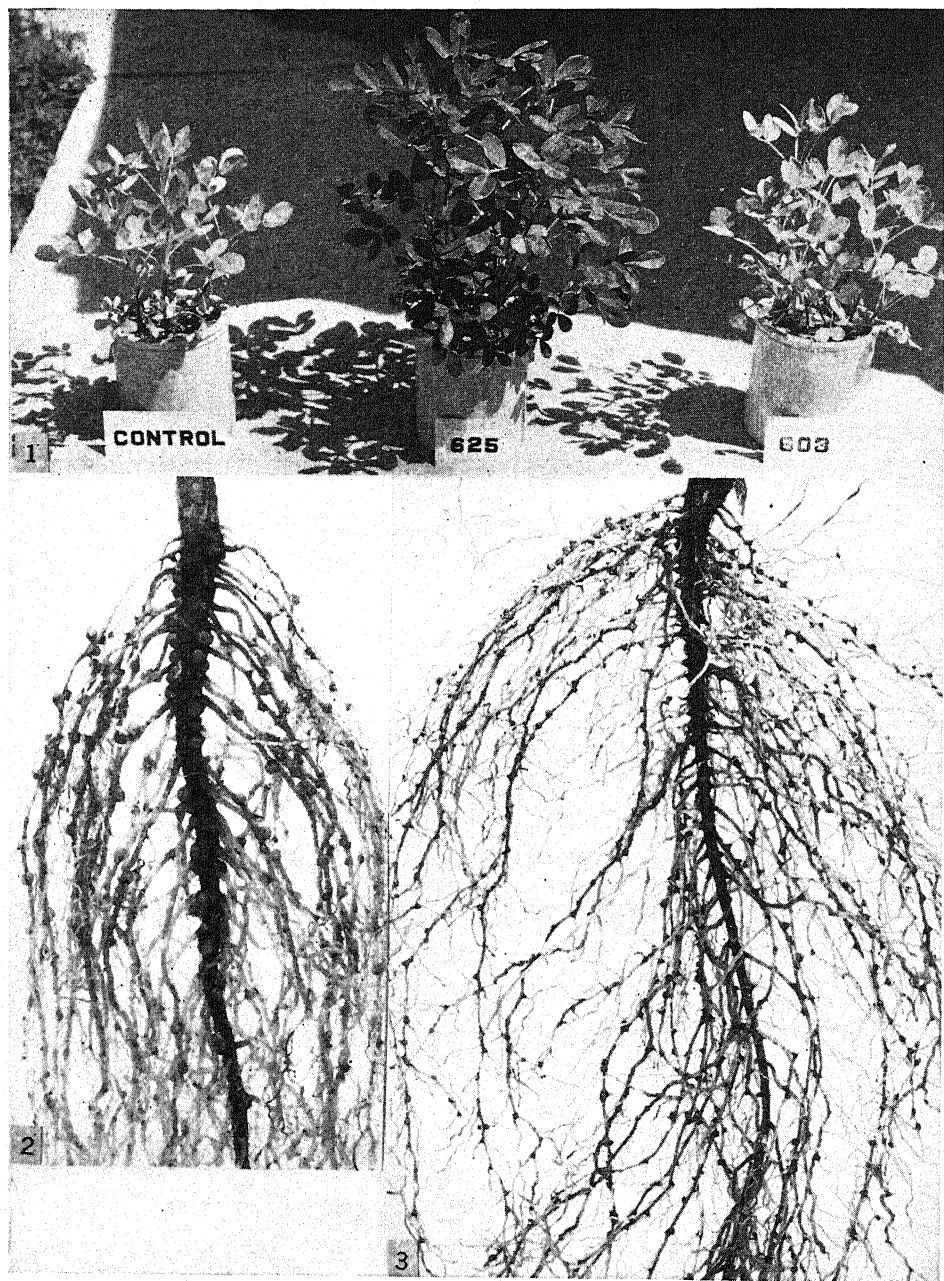
In general the nodules occurred singly, remained simple and spherical, and were similar to the lupine type, inasmuch as there was always a rather broad basal connection with the root, indicative of continued pericambial activity. All nodules (figs. 2, 3), irrespective of their size and source of inocula, were located in the root axils. This arrangement was conspicuous since the root system of this species consists of a central taproot, along which the lateral roots arise in an orderly alignment of four vertical rows, owing to the tetrarch pattern of the xylem. On no occasion were nodules found randomly dispersed along the roots in the arcs between

the protoxylem points, nor did they occur in the regions opposite the protoxylem points where lateral roots had not emerged.

Good agreement was obtained between the growth response of the plants and the size and location of the nodules (figs. 1-3). The strains of rhizobia could easily be divided into three groups on the basis of effectiveness. Although the plants proved rather susceptible to nodulation, as evidenced by the fact that each of the strains produced nodules, only twenty-six strains benefited plant growth. Seventeen of these, as designated in group A, markedly stimulated plant growth, while nine others were beneficial to a lesser extent. The plants nodulated by these latter strains (group B) were significantly better than those of the control and group C series, but differed appreciably from those in group A in regard to dry weights and percentages of fixed nitrogen. There were no differences, however, in the types of nodulation produced by the effective strains (groups A and B), since comparatively large nodules, more or less concentrated on the central upper portion of the root system, were common on all the plants showing green foliage and enhanced growth. The strains comprising group C were definitely non-beneficial, as shown by a stunted, sickly, yellow-green condition of the plants and the occurrence of small nodules which tended to be inconspicuous in the axils of the secondary and tertiary rootlets. The nodules produced by these strains were analogous to those obtained by CARROLL (7) on peanut plants when strains of rhizobia of *Lespedeza*, soybean, cowpea, and velvet bean were used as inocula. In a few experiments conducted during the summer months, plants nodulated by certain of the strains in group C were inferior to those in the control or uninoculated series.

In addition to the small ineffective nodules common to the roots of the peanut, a proliferation comparable in external appearance to the collar type of nodule (41) was frequently noted. This condition is faintly visible in figure 3. To the naked eye these nodose formations were similar to young nodules, although when immersed in water they appeared as bulbous conical swellings completely surrounding the basal portions of the fibrous rootlets. Where two small nodules occurred simultaneously, one in the upper and the other in the lower axil of the rootlet, it was somewhat difficult to distinguish them from the collar or nodose condition, although a morphological differentiation was usually possible by means of a hand lens. All attempts to isolate rhizobia from these swellings were unsuccessful. Histological examination showed them to be merely hypertrophied parenchymatous tissue containing heavy deposits of starch. Since these formations showed no evidence of infection, did not contain bacteria at any stage of development, bore no anatomical similarities to nodules, and were found only on uninoculated plants and those nodulated by the ineffective strains (group C), it is thought they were abnormal proliferations related in some way to nitrogen deficiency. It is likely that a confused interpretation of these abnormal proliferations





FIGS. 1-3.—Fig. 1, response of peanut plant to inoculation with rhizobia: left, no inoculation; center, inoculated with strain 625 (strain 22-2 of University of Hawaii collection), isolated from *Desmodium barbatum* (L.) Benth. and Oerst.; right, inoculated with strain 603, isolated from *Phaseolus lunatus* L. Fig. 2, nodule formation when inoculated with beneficial strain 625. Fig. 3, same when inoculated with non-beneficial strain 603.

in relation to non-beneficial nodules is responsible for the conclusions of RAJU (29) and RAJAGOPALAN (28) that the size and location of nodules on the peanut are not correlated with the effective abilities of the rhizobia.

### Histology and cytology of the nodule

#### MODES OF DEVELOPMENT AMONG THE LEGUMINOSAE

Although the exact mechanism by which the bacteria enter the root is not known, it is generally agreed that the root hairs constitute the normal avenues of infection. It is likely that the rhizobia secrete an enzyme which enables them to penetrate the hair wall; however, cellulose-splitting enzymes have not been detected in cultures of the organisms. Nevertheless, when the rhizobia come in contact with the root hairs, the hair wall is apparently softened or dissolved, curvature of the hair results, and the rhizobia proceed through the hair in a threadlike manner until the basal epidermal cell is reached. At this point the thread frequently becomes multiple, and their penetration into the cortex is accompanied by rapid division of parenchymatous cells. Differentiation within the hypertrophied mass of cells gives rise to the nodule tissues. Other avenues of infection have been known to occur. As early as 1888, BRÉAL (4) was successful in securing infection by piercing the roots of seedlings with a needle which had been dipped in a culture of rhizobia. Other investigators (6) have shown the possibility of rhizobia entering directly through epidermal cells of young root tips, especially where the plants are cultured in artificial media (such as agar) which ordinarily suppress root hair production.

The extent of penetration of the infection threads and the tissues primarily stimulated are important factors in determining the site of nodule origin. THORNTON'S (34) suggestion that the exact location may differ according to the species of host plant seems rather pertinent. In nodules described as exogenous, infection has been limited to the parenchymatous cells of the cortex, and the endodermis and those tissues within the central stele of the root are not directly concerned in their formation other than in the production of the vascular system necessary in the later stages of nodule development. Development of nodules of this type has been described in detail by FRED, BALDWIN, and MCCOY (12), THORNTON (34), and BIEBERDORF (3). On the other hand, VAN TIEGHEM and DOULIOT (38), PARATORE (24), PEIRCE (25), WHITING (40), and TERBY (33) have described nodules of endogenous origin, a condition wherein the nodules have originated by division of pericyclic cells, usually opposite protoxylem points. Nodules of this type have provoked many complications, owing to their similarities to lateral root formation. In general the data offered in support of endogenous origin have not been so convincing or so complete as those presented for exogenous origin; consequently

many phases of their development have not been made clear. It is hoped that certain of these aspects will be clarified by the following observations of endogenous nodule formation.

### Material and methods

Most of the plant material used in the histological study was obtained from the afore-mentioned culture experiments. Occasionally seedlings were cultured in moisture dishes when material was necessary for the detection of root hairs and young nodule stages. In the later phases of the study, field material was obtained for the purpose of comparing the results obtained from sand-grown and soil-grown plants. Fixation of the root material was made at various intervals of time so that successive stages of development could be noted. Frequently entire root systems were fixed in the hope of finding the primary stages of infection. Care was always taken to see that the nodules were not detached, in order that histological sections could be made through the juncture.

Four fixatives were used: Bensley's, Flemming's medium, Wallin's, and formalin-acetic-alcohol. The first three were equally satisfactory in the detection of embryonic nodular and rootlet development, since the osmic acid blackened those areas having concentrated protoplasm and chromatin materials. Use of the microscope (low power objective) or hand lens made it possible to detect these areas prior to their emergence through the root cortex. Flemming's fixative, modified either by decreasing the amount of acetic acid to about 10 drops per 100 cc. or by omitting it entirely, was the most satisfactory solution for routine use. Bensley and Wallin's fixatives were especially useful in differentiating mitochondria and bacteria; while the formalin acetic alcohol, the least satisfactory, was used only when a comparative study of the anatomical features of the tissue was primarily desired. All material was fixed 24-48 hours (depending upon the size of the plant portions), washed, dehydrated, imbedded in 58° paraffin, and cut in serial sections 3-10  $\mu$  thick.

One of the most satisfactory techniques for the study of cellular contents was the placing of surface-sterilized nodules in drops of sterile water on clean glass slides and cutting them in halves. One-half of a nodule was then placed in a certain fixing solution, while the characteristics of the other half were studied by stained smears or in wet mounts. When the major portions of a nodule were treated by different fixatives, the cellular contents in the drop of water or thin free-hand sections through the center of the nodule served for examination of the fresh material. Various staining methods were used to bring out certain details. Flemming's triple, Heidenhain's iron-alum haematoxylin with and without counterstains, Bensley's stain for mitochondria, and MacCallum's modification of the

Gram stain were found satisfactory. Heidenhain's haematoxylin without a counterstain was usually preferred, since uniformity resulted because of the more sensitive control of the staining procedure.

### Discussion and results of histological study

No significant differences were noted in the histology and the cytology of the nodules produced by the effective and ineffective strains of rhizobia. Similarly there were no differences in the structure of the nodules produced on plants of the two varieties. Consequently the following observations pertain in general to the development of nodules, irrespective of these factors.

### MODE OF INFECTION

It became apparent in the initial phases of the histological study that infection of the roots by rhizobia was complicated by several conditions uncommon to other members of the Leguminosae heretofore studied. Although the roots of the peanut are of the taproot type, the distribution and occurrence of the root hairs were unusual. Instead of the hairs being uniformly common and abundant in the regions of elongation of the central and lateral roots, they were extremely rare. When lateral or tip hairs were observed they were few in number and arose at random from widely separated epidermal cells. These findings were contrary to expectation, since it was assumed that the plant would normally have abundant root hair formation, owing to its semi-xerophytic tendency and ability to grow well in warm, loose sandy soil. Such an unusual condition probably accounts for the reports of RICHTER (according to REED, 30) and PETTIT (26) that hairs are entirely absent on peanut roots. On the other hand, tufted clusters or rosettes of hairs, such as noted by WALDRON (39) and REED (30), were frequently found in the root axils. These hairs were less fragile than those common on the lateral surfaces of other leguminous roots, had moderately thick cell walls, and averaged 2-4 mm. in length. They were found on all plants of more than 2 weeks' growth and under all conditions of culture.

Little explanation is given for the presence of the hairs in this unique arrangement. WALDRON (39) found their growth stimulated by high temperature and humidity, but none of the environmental factors he employed explained their presence in the root junctions and the absence of the normally expected lateral type. His conclusion, that lateral hairs are absent because of the loose arrangement of the epidermal and cortex cells, which "seem readily to fall apart like so many poorly cemented bricks becoming loosened," was not confirmed in this study. The root systems of all the plants were white, well developed, and showed no tendency to slough off the cortex layer of cells. It is probable that root hair production was stimulated in the axils as a result of tissue injury and retarded epi-

dermal growth caused by rootlet emergence. In nodulated plants further stimulation of the cells in the axillary locations was undoubtedly provided by subsequent formation of the nodules which, as will be shown later, arose from within these regions and pushed outward through the ruptured tissues. These observations are of interest since SNOW (31) and others have shown that root hair production is commonly stimulated at the site of wounding and especially in those areas where elongation of the epidermal cells has been arrested owing to abnormal swellings. Inasmuch as lateral root hairs occurred infrequently throughout the various methods of plant culture, it is believed that their presence in whorls or tufts around the base of the side roots is perhaps one of inherent, normal nature with this species, rather than a result of the particular environment.

Since root hairs and nodules were found only in axillary regions, it might be expected in the light of previous data (12) that formation of the latter resulted from infection of the former. Such was not shown to be the case. In a series of experiments in which the development of nodules and root hairs was closely followed, none of the data were compatible with the idea that the root hairs participated in the infection process. In the majority of instances where nodules and root hairs occurred in the same axils, hair formation was simultaneous with, or subsequent to, the first indications of nodule development. As the nodules produced by the effective strains enlarged, there was a tendency for the hairs to become less conspicuous; in many cases they were entirely absent. On the other hand, in the presence of the non-beneficial nodules the hairs tended to be persistent. There was close agreement between the presence of the whorled tufted hairs in the axils and the "nodose" condition on the plants showing nitrogen lack. Frequently the hairs had their origin in the epidermal cells of these hypertrophied areas. In several experiments seedlings showing a prevalence of axillary hairs were immersed in suspensions of rhizobia and in their filtrates. On no occasion was curvature or the presence of bacteria observed in the root hairs. This was also true when the randomly scattered lateral hairs were examined. Apparently the rhizobia lack the ability to effect entrance through the hair wall.

The mechanism by which the rhizobia enter the roots becomes a matter of conjecture, since definite data in the affirmative were not obtained. BEIJERINCK (2) and MCCOY (19) have suggested the probability of infection through the ruptured tissue of the root at the time of rootlet emergence. It appears likely that the peanut plant is an example, since nodules did not occur in regions other than the root axils. Further evidence validating cortical splits as avenues of infection was provided by the production of nodules in the axils of adventitious roots emanating from stems which had been covered with soil above leaf branches. On no occasion were nodules formed on the adventitious roots per se, nor did root hairs occur in their junction with the stem. The nodules produced in these axils were identical in

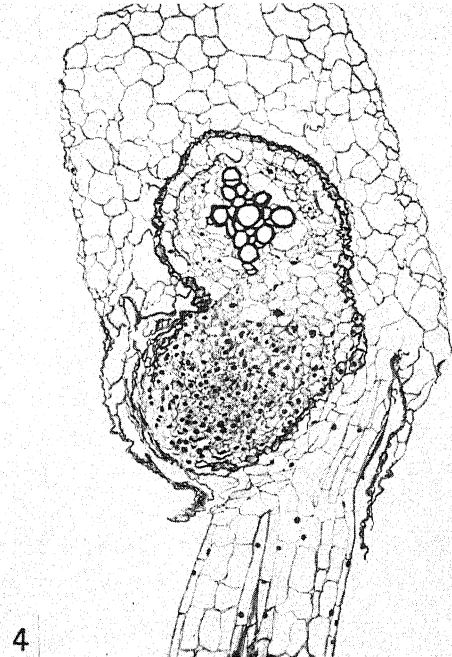
every respect with those formed on the root systems. Various chemical, physical, and mechanical factors common to sand and soil culture undoubtedly facilitate infection through the ruptured tissues.

#### DIFFERENTIATION OF NODULE TISSUES

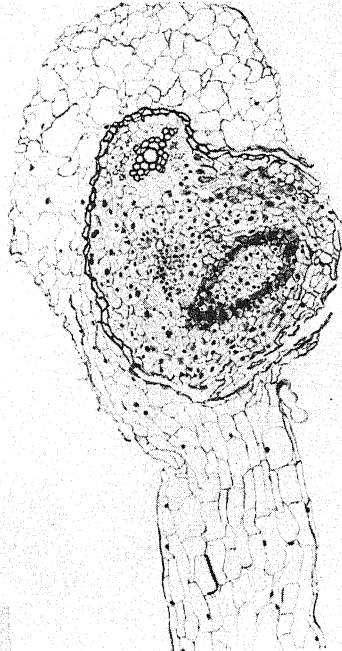
The earliest evidence of nodule formation was a meristematic activity in the pericyclic cells adjacent and in juxtaposition to the same protoxylem strand from which a rootlet had emerged. Students agree that lateral roots also arise in this layer of cells. Fixed preparations of root systems of young inoculated plants showed nodule meristems in the axillary positions about 2 weeks after rootlet emergence. The fact that these areas were not apparent prior to emergence of the rootlet is further indication that the ruptured tissues provided entrance for the rhizobia. The meristems were characterized by small, irregular-shaped, thin-walled cells containing dense cytoplasm, a large nucleus with a fine, evenly granular karyolymph, and one prominent homogeneous nucleolus. As the infected area enlarged by rapid cell division, the densely packed cells tended to push outward and form a conelike proliferation projecting away from the central stele of the mother root. Throughout this stage growth activity remained within the endodermis, which expanded accordingly. Figure 4 shows the location of this meristem during early stages of nodule development.

Occasional mitotic figures and evidences of recent cell division were frequently discernible in the cells surrounding the center zone of growth. The activity of these cells did not appear at first to be affected by the presence of rhizobia within the cytoplasm, although, as the cells attained their maximum size and became filled with bacteria, mitosis diminished and finally ceased. Small vacuoles were not common in the young infected cells; on the other hand, large central vacuoles were prevalent in mature stages. The nucleus and the nucleolus persisted in the cytoplasm of the infected cells until the latter became filled with rhizobia and by-products of the symbiosis. With the production of a central vacuole and increased tension in the cytoplasm, the nucleus was pushed to one side, where it assumed various irregular, crescent, or amoeboid shapes prior to its disintegration.

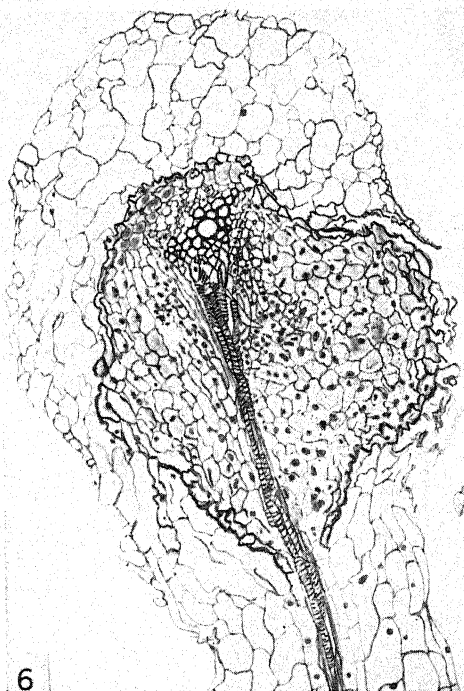
Dissemination of the rhizobia, or the spread of infection, within the bacteroidal area occurred during mitotic division of meristematic cells, as previously shown by LECHTOVA-TRNKA (15). The mode of spread, however, did not appear to be dependent upon the polar attraction of the rhizobia, as described by MILOVIMOV (21) in the lupine nodule. In general the process was analogous to the condition in the bean nodule (19), inasmuch as the rhizobia were more or less passively divided with the cytoplasm as the cell plate was formed between the daughter cells. The efficiency of this mode of bacterial spread was evidenced by the fact that in mature nodules the central bacteroidal area appeared as a solid mass of densely



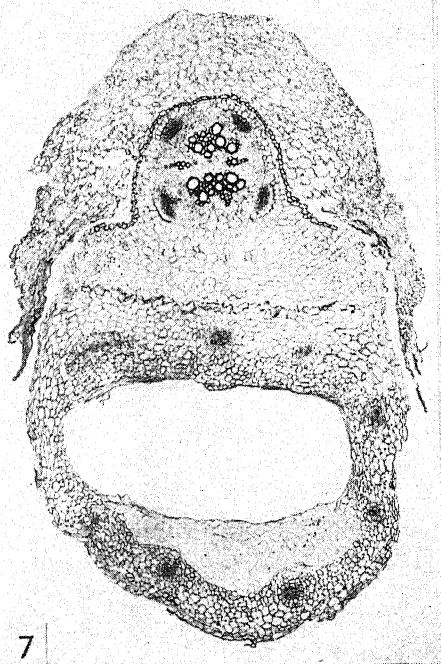
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FIGS. 4-7.—Fig. 4, cross section of primary root and nodule, and longisection through lateral rootlet, showing embryonic nodule (meristematic area) inclosed within endodermis. Fig. 5, later stage showing exit of nodule from root parenchyma, formation of cambial ring, and differentiation of tissue. Fig. 6, tangential section through same nodule and median longisection through lateral rootlet showing close relationship between rootlet and axillary nodule. Fig. 7, cross section of primary root and old nodule prior to complete disintegration. Note the eight vascular bundles in nodule cortex, remnants of bacteroid area below hollow center, and cortical sheath of mother root.

filled cells not unduly hypertrophied as a result of infection. Single or clustered uninfected cells were not common, although zones—each consisting of two or three rows of empty parenchymatous cells—occasionally extended from the periphery into the center of the bacteroidal area. It is likely that these were cells not infected when initial cell division occurred. On no occasion were infection threads or zoogloal strands noted as agents in the transmission of organisms to cells within the bacteroidal area.

In the embryonic nodule the primary mass of undifferentiated meristem showed cell division taking place in all planes. This resulted in a bowl-shaped or spherical proliferation, in contrast to the type of growth described by SPRATT (32) and others (3, 25, 34), where a meristematic cap at the distal end of the nodule causes it to increase in length and become cylindrical. The endodermis continued to envelop the proliferation (fig. 4). The nodule mass usually made its exit from the root tissues about one week after infection. At this time differentiation within the embryonic area was evidenced by the production of a cambial sphere which in cross section appeared as a ring of small, heavily stained meristematic peripheral cells several layers in thickness. This ring (fig. 5) functioned by producing two kinds of parenchymatous cells: to the outside—loosely packed, irregular-shaped cells containing little cytoplasm which served as nodule cortex; and to the inside—cells containing considerable cytoplasm which comprised the bacteroidal tissue. As growth and enlargement of the cells in the bacteroidal area took place there was consequent enlargement of the cambial sphere. During the initial stages of development of the bacteroidal area the cells were characterized by a dense cytoplasm, occasional vacuoles, central location of the nucleus, and inclusion of plastid-like bodies.

In regard to emergence, the peanut nodule simulated a rootlet in its ability to push or digest its way through the ruptured tissues of the mother root. In this respect it differed markedly from the nodules heretofore described on other leguminous plants. In all material examined the fragments of the mother cortex were retained loosely, in the manner of a partial sheath around the base of the emerged nodule (fig. 7). The fact that the nodule lacks a cortical covering provided by the mother root tissue becomes an important characteristic, since nodules of the exogenous type always remain inclosed within a layer of root cortex. About the time the nodule effected its exit through the root parenchyma, a periderm layer was formed along the exposed periphery from the collapsed and compressed cells of the nodule cortex. Since in roots the first true periderm commonly arises in the outer layers of the pericycle (10), its presence as a peripheral layer of the nodule serves as further evidence of its endogenous or pericyclic origin.

It was not until the nodule became macroscopically visible outside the root cor-



tex that vascular connection between the xylem and phloem of the root and nodule was evident. Development of the vascular tissues in the peanut nodules is apparently later than in nodules of cortical origin. Recently BIEBERDORF (3) has shown that conductive tissue in the soybean nodule may become noticeable soon after the rhizobia have penetrated the first two layers of the cortical parenchyma. The delay in vascular formation in the peanut nodule is probably accounted for by the inclusion of the meristematic area within the endodermis, where transportation of the nutrients and by-products between the nodule and the central vascular system of the root is facilitated by absorption and osmosis. The first evidence of the vascular system was a small meristematic area in the nodule cortex about midway between the cambial ring and the protoxylem strand (fig. 5). As division of the cells continued, the new walls were laid down more or less parallel to the radius of the root, and the xylem and phloem elements became differentiated in a tetrarch arrangement. These elements connected at right angles with the protoxylem strand, from which the nodule and the lateral root originated. On no occasion could vascular strands in a single nodule be traced to more than one primary xylem group, such as described in nodules of *Medicago* and *Vicia* (5, 36). The major conducting unit of the vascular system emanating from the central stele of the root encircled the nodule by means of four branches which developed at right angles to one another from the four protoxylem points at the nodule base. These branches subsequently divided, since eight vascular bundles were frequently seen in cross-section preparations of the mature nodule (fig. 7). These observations are in agreement with those of SPRATT (32), who was among the first to show a relationship between the xylem arrangement in the stele of the root and the vascular branching in the nodule.

Nodules have commonly been classified on the basis of morphology. In those characterized as compound, elongated, or cylindrical, growth always results from an apical or cap meristem. Thus as the nodule mass enlarges, the basal connection with the root tends to remain constant and thereby appears constricted. In consequence PEIRCE (25) assumed that as growth continued, the vascular connection between the nodule and the root became limited in function because of lack of secondary thickening at the nodule base, whereas the demands upon the conductive system tended to augment. He concluded that in such nodules the vascular system became inadequate and effected more rapid degeneration and death of the nodule cells. In comparison, peanut nodules occur singly and remain simple spherical formations. A broad basal connection with the root is maintained as a result of the prolonged cambial activity. In all the material examined the vascular connections between the nodules and the roots appeared adequate and normal until degeneration of the bacteroidal area was in an advanced stage.

BACTERIA, MITOCHONDRIA, AND OTHER INCLUSIONS  
OF BACTEROIDAL CELLS

Despite the fact that nodule tissues provide an excellent opportunity for studying the effect of the microorganisms upon the plant cells, and vice versa, it is not always possible to follow the changes occurring in the infected cells. In some instances difficulty arises from the rapidity with which the changes take place; in others, from the inability of methods to make clear the intricate transformations. In the peanut nodule the absence of infection threads or zoogloeal strands and the marked density of the cytoplasm in the small meristematic cells made it difficult to study the preliminary changes taking place in the infected cells of the bacteroidal area.

In general, fixed tissue preparations did not afford an acceptable medium for study of the rhizobia. Occasionally short, rod-shaped bacterial bodies, averaging  $1\ \mu$  by  $0.5\ \mu$ , were discernible along the periphery of newly infected cells, but the morphology and identity of these forms became less definite in later stages. Typical Gram negative rhizobia were always easily demonstrated in stained smears or wet mounts of a crushed nodule suspension. The young or physiologically active forms stained uniformly with weak carbol fuchsin (Ziehl formula diluted 1-10 with water) and other ordinary methods. With aqueous solutions of the anilin dyes the older or mature forms of the rhizobia appeared slightly swollen and showed uneven or banded areas within the bacterial cytoplasm. These banded areas alternated with one to three refractile bodies per cell, shown by the alpha-naphthol-phenylenediamine test of DIETRICH and LIEBERMEISTER (9) to be fat inclusions. These findings are in agreement with those of LEWIS (17), who demonstrated the fatty nature of the refractile inclusions in bacteroids of other strains of rhizobia.

Mitochondria were conspicuous in the young meristematic cells prior to formation of the cambial ring, although as the bacteroidal area became more mature, they were obscured by the other cellular contents. Their presence is generally interpreted as evidence of cellular stimulation caused by infection, or of an increased activity of the cells resulting from active division. Nodule tissues, fixed and stained, provided the only medium for examination of these bodies, since it was impossible to demonstrate them in wet mounts and stained smears owing to their fragility. Bensley's technique showed mitochondria in young infected cells of the bacteroidal area as reddish spherical bodies measuring about  $0.5\ \mu$  in diameter against the cytoplasmic background counterstained by methyl green. Heidenhain's stain showed less differentiation, owing partly to simulation of the fat inclusions of the rhizobia to mitochondria, since both retained the haematoxylin more densely than the remainder of the bacterial cell.

The most conspicuous inclusions of the infected cells in the bacteroidal area

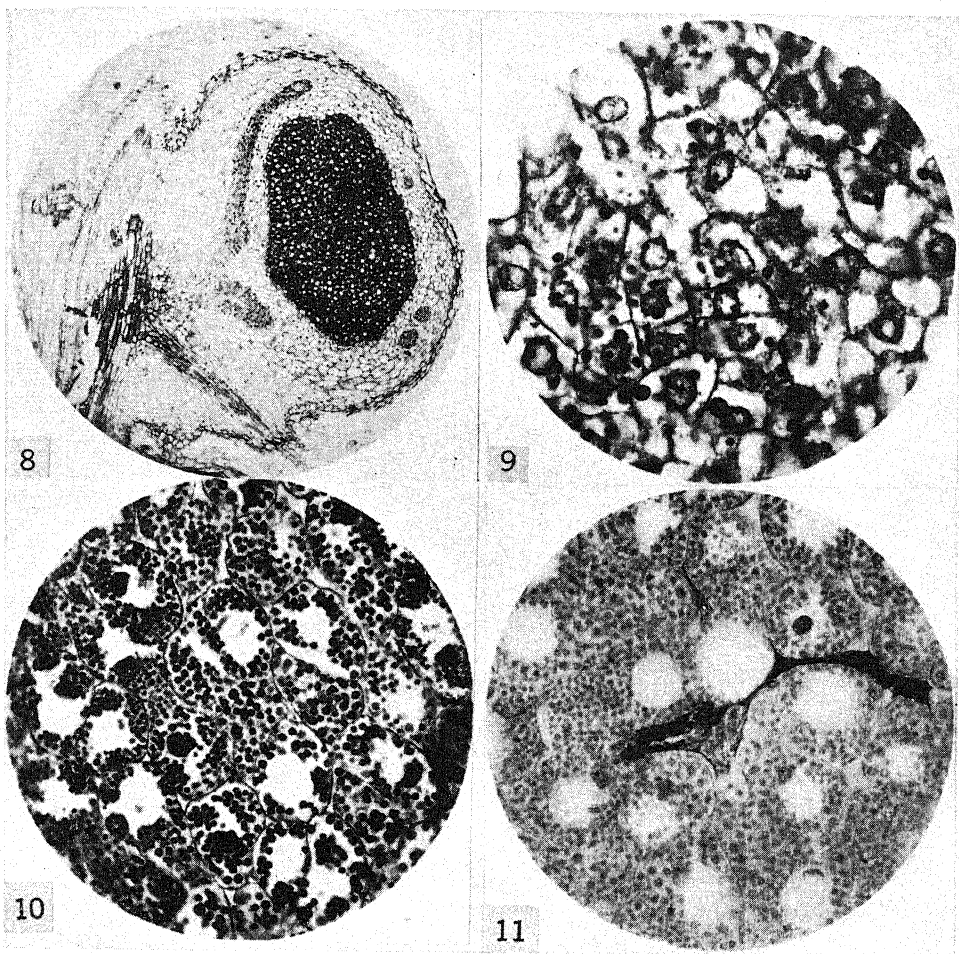
were spherical, plastid-like bodies which averaged  $3\ \mu$  in diameter. These bodies first appeared singly and dispersed throughout the young infected cells of the centermost region of the nodule mass (fig. 9). On no occasion were they found in the meristematic cells, in the nodule cortex, or in the root tissues. As the cells in the bacteroid area enlarged and became mature, the number of these spherules per infected cell increased rapidly. Frequently more than fifty such bodies appeared in cross section of a single cell. They stained heavily and occupied much of the cell space between the central vacuole and the cell wall (fig. 10). By the time differentiation of the tissue within the nodule was complete, the spherical bodies were so numerous that the mitochondria and rod-shaped bacteria could not be recognized with certainty. Apparently LECHTOVA-TRNKA (15) observed the same phenomenon, although she interpreted these bodies as large spherical forms of the rhizobia which had evolved from the rod-shaped stage. She assumed that the change in morphology was so rapid that all the rod forms in the cells were transformed into spheres at approximately the same time.

Various microchemical tests and staining methods were employed to identify these inclusions. In fresh, unstained wet mounts the bodies were refractive, colorless, and homogeneous. Reduced light or dark-field illumination showed the bulk of each body inclosed by a thin membrane and frequently containing several granules or one angular central granule in the ground matrix. LECHTOVA-TRNKA (15) suggested a likeness of these granular centers to nuclei, inasmuch as they occupied an appropriate position in the spherical forms which she assumed to be rhizobia. In general the spheres occurred singly, although a diplo arrangement was occasionally noted; however, when slight pressure was applied to the coverslip preparation, the joined bodies always separated with ease. The inability of these bodies to multiply was evidenced by no increase in numbers when sealed wet mounts were examined over a period of several weeks. Similarly there was no tendency toward disintegration or changes in form and size under these conditions. On the other hand, the number of rhizobia in the same suspensions was greatly augmented.

The inclusions gave negative reactions to tests specific for starch, glycogen, cellulose, pectin, tannin, and resins. The supposition that they were fat globules or involution forms of rhizobia undergoing fatty degeneration was not verified by the use of (a) Herxheimer's alcoholic-acetone solution of Sudan III in conjunction with a methylene blue counterstain, (b) Eisenberg's iodine-basic-fuchsin, (c) Dietrich and Liebermeister's naphthol blue method, and (d) Michaelis' Sudan IV. On the other hand, the rhizobia in the same microscopic fields showed positive fat tests, thereby providing a control on the technique. The bodies were unchanged after treatment with acetone, ether, and chloroform.

The spherical bodies from the fresh nodule material were insoluble and slightly swollen in dilute acetic acid. Their proteinaceous nature was established by weakly

positive reactions with the xanthoproteic test and Millon's reagent. Pronounced positive results were obtained in all tests with Krasser's methods as outlined by ZIMMERMANN (42) and ECKERSON (11). The fundamental mass of the spherical



FIGS. 8-11.—Fig. 8, longisection of mature nodule showing junction of vascular system of nodule and that of lateral rootlet with primary stele. Fig. 9, young bacteroidal cells showing rhizobia and beginning of aleurone formation. Fig. 10, bacteroidal area of mature nodule showing cells packed with aleurone. Fig. 11, same of old nodule showing dissolution of cell walls, disintegration of aleurone, and rhizobia packed in intercellular spaces.

bodies stained dark red with yellow crystalloid centers when thin sections of fresh nodule material or dried smears of crushed nodule suspensions were flooded with an alcoholic solution of picric acid and stained with alcoholic eosin. Similarly, the amorphous ground substance stained blue and the crystalloid center appeared

yellow-green after being treated in an alcoholic solution of nigrosin and picric acid. In consequence, these bodies were identified as aleurone or protein grains.

A rather marked affinity for a number of staining solutions was shown by the aleurone in smear as well as in tissue preparations. Variations were noted in the ability of the apparently young and old bodies to take the counterstain of the Gram method (Kopeloff and Beerman modification), but there was never any transition from a Gram negative character. The narrow zone between the membrane of the aleurone body and the internal matrix was most satisfactorily shown with Loeffler's alkaline methylene blue. This might have been due to the shrinkage of the amorphous protein elements by the alkaline solution. They were unstained against a faint blue background by the negative method of Barlow's crystal violet technique. The granules or globoid elements within these protein bodies were especially conspicuous when treated with a 1 per cent solution of aqueous erythrosin or with Ziehl's carbol fuchsin. There was no evidence of acid-fast properties. The aleurone bodies were stained a deep uniform red by Dorner's spore technique.

Flemming's triple stain afforded the best means of differentiating the aleurone from the cellular structures in tissue preparations. The cytoplasmic remnants and walls of the plant cells stained orange, the nuclear fragments dull red, and the nucleolus brilliant red, while the aleurone bodies appeared as granular, vacuolated, rose-red inclusions. Starch grains which stain a deep blue by this technique were rarely seen within the bacteroidal cells, although they were frequently observed in the innermost layers of the nodule cortex. With Heidenhain's solution the aleurone in young nodule tissues stained with the same intensity as the nucleolus, whereas in old material it possessed little affinity for the dyes and appeared homogeneously granular or vacuolated (fig. 11). Wallin and Bensley's techniques gave erratic results. In young peripheral cells near the cambial ring a majority of the aleurone bodies showed marked affinity for the acid fuchsin; in old cells of the bacteroidal area of the same nodule they stained green. In many instances both conditions were exhibited in the same cells.

LECOMTE (16) appears to have been the first to note the presence of aleurone in nodules of the peanut plant, although he credited GODFRIN (13) with priority.<sup>1</sup> LECOMTE regarded this substance as a rich nitrogenous reserve and assumed that it played a definite role in the symbiotic nitrogen-fixation process. To date, the presence of aleurone in nodules of other species of the Leguminosae has not been reported. Various considerations may be given to the origin and nature of the aleurone grains and their function in the root nodules of the peanut plant.

<sup>1</sup> LECOMTE (p. 304) inadvertently substituted in his quotation of GODFRIN's work (p. 32) the words "des tubercules" for GODFRIN's "de ce cotylédon." No attention was given by GODFRIN to the study of root nodules.

Aleurone is a common ergastic proteinaceous storage product in seeds of such plants as the castor bean, lupine, corn, etc. In different species it varies considerably in color, form, and structure. In seeds of *Pisum* the aleurone grain consists only of an amorphous substance; in others the ground substance incloses a rounded globoid particle, a crystal of calcium oxalate, or a large angular albuminous crystalloid. The best known aleurone bodies are probably those of the deeply lying endosperm cells of the castor bean, which consist of a protein ground substance, a crystalloid, and a globoid composed of a double phosphate of calcium and magnesium together with certain organic constituents.

Early investigators have shown that aleurone grains are formed in vacuole-like cavities as the seed matures. DANGEARD (8) was among those to emphasize these cavities as actual sap vacuoles, since he maintained that the vacuolar material passes through a reticular stage and breaks up into a number of small vacuoles in which the gradually condensing constituents differentiate as crystalloid, globoid, and ground mass. MOTTIER (23), however, has claimed that aleurone formation involves the activity of permanent plastid primordia, which aggregate in large numbers in the vacuole-like cavities, where their combined products unite to form the aleurone grains. Accordingly he has assumed the primordia for the aleurone bodies to be mitochondria-like, and their formation analogous to that of leucoplasts and chloroplasts from plastid primordia (14, 18, 20, 22).

The reports of these investigators are of interest in connection with the observations made in the course of this study. It seemed apparent from the initial appearance of the aleurone grains in the infected cells of the peanut nodule that there was a relationship between their formation and the presence of mitochondria. With the onset of aleurone formation there was always a decrease in the numbers of mitochondria accompanied by a clearing of the cytoplasm. In the mature cells of the bacteroidal area mitochondria were not distinguishable; whether this was due to their complete disappearance or to their being masked by the cellular contents of aleurone bodies could not be determined. The consistent occurrence of aleurone in the cytoplasm between the central vacuole and the cell wall and the absence of the many small vacuoles so conspicuous in clover and alfalfa nodules lessen the likelihood that their formation was concerned with vacuole-like cavities. The fact that aleurone was formed only in the infected cells similarly suggests a relationship with the rhizobia. It is believed that this relationship differs from that involving starch production, however, since MCCOY (19) found that starch deposits occurred primarily in the uninfected bacteroidal cells of the bean nodule.

Aleurone grains were sparse in young nodules from plants undergoing nitrogen lack, although in nodules showing approximately the same stages of development—taken from plants showing greening of the leaves—they were common. Yet a sig-

nificant difference between their numbers per cell in the mature nodules produced by the effective and the ineffective strains was not obtained. Little explanation can be offered for the presence of aleurone in nodules of the peanut plant and its apparent absence in nodules on other leguminous species. The fact that aleurone grains produced in certain species are soluble in water and in others are insoluble should not be overlooked. It is probable that the chemical complex of aleurone is affected by the physiological nature of the peanut plant, since oils occur in such large quantities. Such a condition may account for its insolubility.

Disintegration of the aleurone bodies, as evidenced by their mottling, irregularity in shape, and diminution in size, was apparent in all sections of moderately old nodules. As disintegration progressed, the bodies contained circular or angular cavities in lieu of the previous granular or crystalloid centers. Differences in the appearance of the aleurone found in mature and old nodules is noted by comparing figure 10 with figure 11. In general these results are in agreement with the observations of LECHTOVA-TRNKA (15) in regard to the "spherules," which she assumed to be large involution forms of rhizobia, and also with GODFRIN'S (13) description of aleurone dissolution in cotyledons of the peanut plant. The probability that aleurone is ultimately transformed by enzymes produced by the rhizobia is not to be excluded. Old empty nodules, the bacteroidal contents of which had undoubtedly been absorbed by the plant, were frequently found adhering to the roots. In many instances these old shells were totally devoid of cracks or crevices through which the contents might have exuded into the soil. On no occasion were hollow-center nodules found on the plants inoculated with the strains of group C. It is likely that, since the ineffective nodules are minute and since they apparently undergo complete disintegration in a relatively short time, the old forms escaped observation at the time of sampling. THORNTON (37) has recently commented upon the rapidity with which the ineffective nodules of red clover disintegrate.

#### DEGENERATION OF NODULE

One of the first indications of nodule disintegration was the production of a layer of cells with heavy suberized walls at the base of the nodule, more or less perpendicular to the main tube of the vascular strand entering from the root. This suberized cell layer encroached upon and later severed the central vascular system of the nodule. Thereafter the vascular bundles in the nodule cortex lost many of their staining properties, and the xylem and phloem elements became less distinct. This suberization (fig. 7), formed between the base of the empty nodule and the mother root, eventually became a seal on the root surface after the nodule had completely decayed. It undoubtedly functions in inhibiting penetration of many contaminating microorganisms into the wound incurred in the root tissues when the old nodule sloughs off. About the time suberization became evident, masses

of rhizobia in zoogloea-like strands appeared between the walls of the cells in the innermost bacteroidal region (fig. 11). Many of these strands extended for considerable distances through the bacteroidal zone and appeared multi-branched when traced in serial sections. The rhizobia in these strands had marked affinity for many of the stains used and were prominent in comparison with the faintly stained background of disintegrating aleurone bodies and cellular contents. According to THORNTON (35), the presence of bacteria in intercellular spaces is due to their attack on the cell walls as a result of an unbalanced equilibrium of the food supply brought about by the inefficiency of the vascular system. The observations in this study lend support to this explanation. In general, necrosis progressed from the innermost area of the bacteroidal zone to the periphery of the nodule, with the result that the plant absorbed the major part of the nodule contents prior to disintegration of the vascular branches and collapse of the nodule cortex. Many old nodules were sectioned wherein only faint remnants of the bacteroidal area remained near the cortical periphery (fig. 7).

Old nodules were recognized by their dark color, wrinkled surfaces, and soft texture. In the early stages of degeneration the bacteroidal areas had a mucilaginous consistency and could readily be lifted out of the cortical covering with a dissecting needle when the nodules were cut in halves. In advanced stages of decay, splits in the nodule cortex—from which there exuded a slimy liquid—were occasionally noted. This exudate consisted of protozoa, filamentous fungi, large bacilli, and an abundance of short, rod-shaped forms typical of rhizobia. It is undoubtedly in this manner that the root nodule bacteria return to the soil.

#### RELATIONSHIP OF NODULES TO ROOTS

Nodules of the peanut plant and lateral roots have the following similarities in structure and development: (a) both arise in the same layer of cells, the pericycle; (b) both points of origin are in the cells adjacent and in juxtaposition to protoxylem points; (c) both structures digest or push their way through the root cortex; (d) during their existence the nodules, as well as the lateral roots, are entirely free of the cortex of the mother root; and (e) in regard to the vascular system the strand supplying the nodule and the lateral root can be traced to only one primary xylem group.

Despite these similarities, it is not to be understood that peanut nodules are modified lateral roots. These data, as well as those presented by others, emphasize the existence of nodules as separate and distinct entities from root structure, since they (a) arise only as a result of bacterial infection, (b) contain a definite bacteroidal area within a distinct cortex, and (c) possess no central stele, no area analogous to a root cap, and no epidermis.



### Summary

1. Each of fifty-nine strains of rhizobia, isolated from a variety of leguminous plants, proved infective upon the roots of the peanut plant. Seventeen strains markedly enhanced plant growth and nine were effective to a less extent, in contrast to thirty-three strains which were decidedly non-beneficial.

2. All nodules, irrespective of size and the sources of inocula, were located in root axils. Those produced by the non-beneficial strains were inconspicuous and tended to be visible only when the roots were immersed in water.

3. Abnormal nodose formations, which proved to be merely hypertrophied parenchymatous tissue containing heavy deposits of starch, were frequently found at the base of the rootlets of plants deficient in nitrogen.

4. Infection of roots results from invasion of rhizobia through the ruptured tissue at the site of lateral root emergence. Lateral or normal root hair formation is rare, whereas tufted whorled rosettes of hairs are common in the root axils. No data were obtained supporting root hairs as avenues of infection.

5. Origin of the nodule is in the pericyclic cells adjacent and in juxtaposition to the protoxylem strand from which a rootlet had emerged. Until such emergence the nodule remains within the endodermis. Dissemination of rhizobia throughout the bacteroidal area is effected by passive transmission of rhizobia to new cells during the laying down of the cell plate after mitosis.

6. Differentiation of the nodule results from the formation of a peripheral meristem which serves in the production of a nodule cortex and a bacteroidal area. A branched vascular system supplying the nodule surrounds the bacteroidal area and connects with the xylem and phloem elements of the main root through a single connecting strand at the base of the nodule.

7. Spherical plastid-like bodies, identified as aleurone grains, were abundant in the infected cells of the bacteroidal area. It is thought that aleurone is directly concerned with the bacteria-plant symbiosis, and is of significance in the nitrogen-fixation process.

8. Initial stages in degeneration of the nodules are characterized by the formation of a suberized layer of cells at the base of the nodule, thereby cutting off the vascular supply. In consequence the bacteroidal area assumed a slimy consistency and rhizobia invaded the intercellular spaces. The bacteroidal area of many nodules was found to be absorbed by the plant prior to collapse of the old nodule cortex.

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# CHROMOSOME CATENATION IN OENOTHERA

K. T. JACOB

(WITH TWENTY-FIVE FIGURES)

## Introduction

The cytology of *Oenothera* is of special importance, since it was in this genus that various correlations between the chromosome content or behavior and genetic phenomena were first described. The first tetraploid mutant (*O. gigas*) was investigated by GATES (16), who suggested that tetraploidy here arose through failure of the chromosomes to separate in the first or early division of the fertilized egg. It was in *O. rubrinervis* that the first instance of nondisjunction was reported (15) and the first instance of triploidy dealt with (17). The first trisomic investigated cytologically (18) also belongs to this genus. Parallel mutations were described in this genus in 1912, and their importance in evolution was pointed out later (19).

GATES (20) listed the chromosome numbers in *Oenothera*, and GATES and FORD (27) recorded the catenations so far investigated. By catenation is meant the linkage of the chromosomes end to end in the prophase of meiosis to form one or more rings, and the opinion that the particular arrangement is fixed for each species or type is now generally held. The maximum catenation is usually the significant one, although derivative associations are observed with variable frequency, probably due to failure of chiasma formation between the pairing bivalents (4). It is well known that in *Oenothera* species with chromosome catenation there is also genetic linkage, so that only two types (Renner complexes) of male and female gametes are generally produced. Also each chromosome in the ring consists of two ends and a central portion, these central portions being different while the two ends of each chromosome correspond respectively with one end of two other chromosomes (7).

The present paper embodies an investigation of the catenations of four species and ten hybrids, three of the hybrids including two types each. The plants were grown by Professor R. R. GATES at the Courtauld Laboratory in Regent's Park, London. To him my thanks are due for kindly supplying the material for this investigation. These are all species, chiefly from eastern Canada, described in a monograph (22), but the first four listed and the male parent in no. 10 are undescribed forms named after their place of origin. The plants are listed in table 1.

## Cytological technique

The cytological material of these species and the hybrids was selected by examining one anther of each bud in a drop of Belling's acetocarmine. The buds were

collected on bright days between 10:30 and 12:00 A.M., and stripped of their sepals and petals, the tops of the anthers being cut off with a sharp razor. They were then dipped in Carnoy's fluid (modified by Semmens) for 1-2 seconds, rapidly rinsed in water, and fixed in Flemming's medium solution. An air pump was used only in those cases where the buds failed to sink, but it was usually unnecessary. Navashin's was also used as a fixative, but not with very satisfactory results.

The fixed material was washed for 12-24 hours in running water and dehydrated by the chloroform method. Sections were cut 14-18  $\mu$  thick. Material fixed in Flemming was bleached before staining in a mixture of 20 volumes of  $H_2O_2$  and 60 volumes of 80 per cent alcohol by keeping it for 4-5 hours near a 60 watt lamp. Chlorine water as well as nascent chlorine was tried for bleaching, but the results were not entirely satisfactory. The slides were stained by the gentian violet-iodine-chromic acid technique. The Feulgen fast green method (45) did not prove satisfactory. These staining methods have all been described elsewhere (32).

The laborious nature of the section method just described impelled the use of a rapid and reliable smear technique (6) in those cultures where the buds were of medium size. By this method about 30-40 slides could be made in a day, but a considerable number had to be discarded owing to desiccation of the pollen mother cells on the slides before fixing. The buds were selected by examining one anther of each in Belling's acetocarmine. Although the chromosomes could not be seen, owing to the granular nature of the cytoplasm, the stage of development of the pollen mother cells could be ascertained by this pre-examination. The ordinary smear method with the scalpel or slide was unsatisfactory, since the anthers were slippery and the juices from the anther wall tissue interfered with fixation and prevented most of the pollen mother cells from adhering to the slide. Best results were obtained by holding the selected bud base downward, after removing the sepals and petals, and cutting off the tops of the anthers with a sharp scalpel. The buds were then squeezed, holding them upright, and the emerging pollen mother cells smeared on to the slide. The slides were immediately plunged into the fixing fluid, prepared as follows:

Solution A:

Chromic acid.....	5 gm.
Glacial acetic acid.....	50 cc.
Distilled water.....	320

Solution B:

Formalin.....	100 cc.
Distilled water.....	275

Equal volumes of solutions A and B were mixed just before use. Fixation took 4-5 hours. The slides were then kept in solution A for about 10 minutes, washed, and stained by the gentian violet-iodine-chromic acid technique.

## Observations

Stages prior to diakinesis were not studied. Table 1 lists the catenations observed.

An attempt was made to fix the relative positions of the nucleolar chromosomes in the ring, but it was not possible in all the plants. The importance of nucleolar

TABLE 1  
LIST OF CATENATIONS

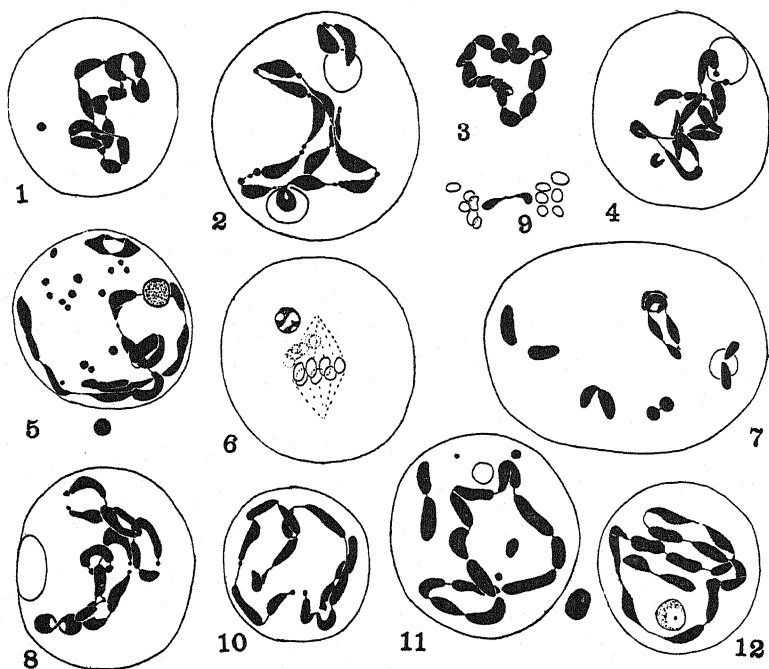
CULTURE NO.*	PLANT	MAXIMUM CATENATION OBSERVED
	Species:	
1.....	<i>O. St. Eustache</i> (Quebec)	⊙ 14
12.....	<i>O. Ile aux Coudres</i> (Quebec)	⊙ 14
13.....	<i>O. Burnham</i> (Somerset, England)	⊙ 10, ⊙ 4
18.....	<i>O. Fort Coulonge</i> (Quebec)	⊙ 14
	Hybrids (F <sub>1</sub> ):	
148.....	<i>O. angustissima</i> Gates × <i>O. angustissima quebecensis</i> Gates	⊙ 8, ⊙ 4, 1 <sub>II</sub>
149.....	<i>O. angustissima quebecensis</i> × <i>O. angustissima</i>	⊙ 8, 3 <sub>II</sub>
150.....	<i>O. hazelae</i> Gates × <i>O. hazelae</i> var. <i>parviflora</i> Gates	⊙ 10, 2 <sub>II</sub>
152.....	<i>O. biformiflora cruciata</i> Gates × <i>O. angustissima quebecensis</i> Gates	⊙ 4, 5 <sub>II</sub>
154.....	<i>O. biformiflora cruciata</i> × <i>O. rubricapitata</i> Gates Type I: flowers broad-petalled Type II: flowers narrow-petalled	⊙ 10, 2 <sub>II</sub> ⊙ 12, 1 <sub>II</sub>
155.....	<i>O. eriensis</i> Gates × <i>O. Long Island</i> (species II)	⊙ 12, 1 <sub>II</sub>
157.....	<i>O. repandodentata</i> Gates × <i>O. eriensis</i> Gates	⊙ 10, ⊙ 4
159.....	<i>O. albinervis</i> Gates (from Fargo, N.D.) × <i>O. rubricapitata</i>	⊙ 12, 1 <sub>II</sub>
160.....	<i>O. albinervis</i> Gates (from Barrie, N.D.) × <i>O. rubricapitata</i> Type I: without red papillae Type II: with red papillae	..... ⊙ 8, 3 <sub>II</sub>
161.....	<i>O. rubricapitata</i> Gates × <i>O. albinervis</i> Gates Type I: without red papillae Type II: with red papillae	⊙ 10, ⊙ 4 ⊙ 14

\* No. under which the plants were grown in the summer of 1938.

studies was first emphasized by HERTZ (30, 31). Earlier, DE MOL (37) found that primary diploids have two nucleoli; triploids, three; and tetraploids, four. Other investigators have subsequently dealt with this problem in other plants, and GATES (25, 26) reviewed the relevant literature.

Figure 4 shows the two satellited chromosomes attached to the nucleolus in the hybrid *O. angustissima* × *O. angustissima quebecensis*. The maximum catenation observed was ⊙8 + ⊙4 + 1<sub>II</sub> (table 1), but in the figure the ring of 8 is broken up into a chain of 7 and 1 univalent. If the positions of the chromosomes in the rings are fixed (24), it is safe to assume that the ring of 8 in this case includes the two satellited chromosomes. Figure 5 shows the nucleolus in association with the chain

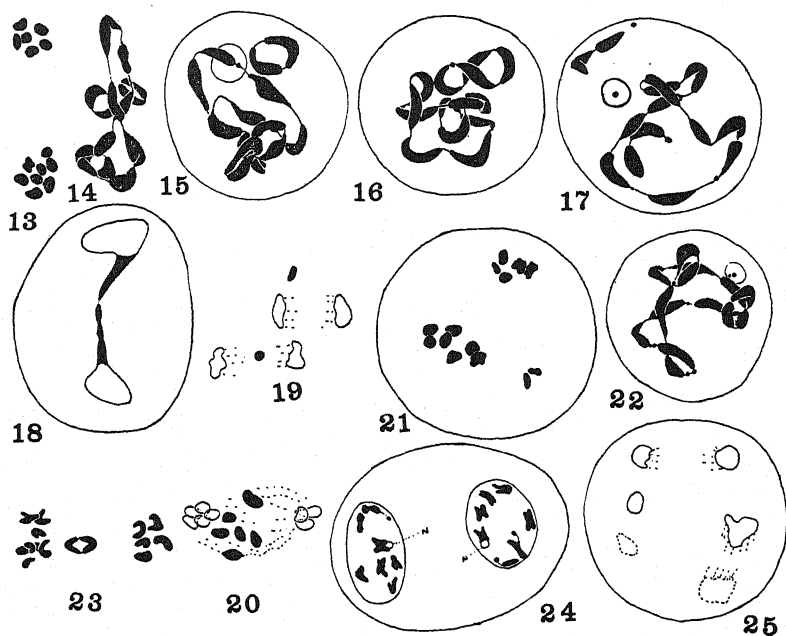
of 8 in the reciprocal hybrid *O. angustissima quebecensis* × *O. angustissima*, where the maximum catenation observed was  $\odot 8 + 3_{II}$ . Here also the nucleolar chromosomes are included in the  $\odot 8$ . Figure 7 shows that the nucleolar chromosomes



FIGS. 1-12.—Fig. 1, *O. St. Eustache*, diakinesis showing ring of 14. Fig. 2, *O. Burnham*, same with ring of 10, 2 bivalents, and a number of tandem terminal granules. (Maximum catenation is ring of 10 + ring of 4. Two nucleoli seen, one on ring of 10 and other on ring of 4.) Fig. 3, *O. Fort Coulonge*, late diakinesis showing ring of 14. Fig. 4, *O. angustissima* × *O. angustissima quebecensis*, 2 satellited chromosomes attached to nucleolus at diakinesis (maximum is ring of 8 + ring of 4 + 1 bivalent). Here satellited chromosomes are included in ring of 8, which is broken up. Fig. 5, *O. angustissima quebecensis* × *O. angustissima*, diakinesis showing chain of 8 + 3<sub>II</sub>. Number of bodies seen inside nuclear membrane and one outside. Fig. 6, same hybrid, micronucleus at metaphase II, formed from single chromosome. Fig. 7, *O. biformiflora cruciata* × *O. angustissima quebecensis*, diakinesis showing ring of 4 + 5<sub>II</sub>; one bivalent attached to nucleolus. Fig. 8, same hybrid, diakinesis showing 16 chromosomes, terminal granules, and interlocking of bivalents. Fig. 9, same, anaphase I showing lagging due to interstitial chiasma. Fig. 10, *O. biformiflora cruciata* × *O. rubricapitata*, type I. Diakinesis showing ring of 10 + 2<sub>II</sub>; terminal granules seen. Fig. 11, type II. Diakinesis showing ring of 12 + 1<sub>II</sub>. Four bodies inside nuclear membrane and one outside. Fig. 12, *O. eriensis* × *O. Long Island* (species II), diakinesis showing ring of 12 + 1<sub>II</sub>.

form a free bivalent in the hybrid *O. biformiflora cruciata* × *O. angustissima quebecensis*, where the nucleolus is found in association with this bivalent. Similarly, in culture no. 160 (table 1), in type II with red papillae, the nucleolar chromosomes are included in the  $\odot 8$  (fig. 15); and in culture no. 161, type I, they are on

the ring of 10 while in type II they are naturally in the ring of 14, since all the chromosomes are linked together (fig. 22). In the *O. Burnham* strain two nucleoli are produced, one attached to the ring of 10 and the other to the ring of 4 (fig. 2). In this case the ring of 4 is broken up into two rod bivalents.



FIGS. 13-25.—Fig. 13, *O. eriensis* × *O. Long Island* (species II), metaphase II showing 8 chromosomes at one plate and 6 at other. Fig. 14, *O. albinervis* × *O. rubricapitata*, type II. Diakinesis showing ring of  $8+3_{II}$ . Fig. 15, type II. Diakinesis showing chain of  $8+3_{II}$ ; one satellite in chain of 8. Figs. 16-21, *O. rubricapitata* × *O. albinervis*, type I. Fig. 16, diakinesis showing ring of  $10+ring\ of\ 4$ . Fig. 17, showing ring of  $10+2_{II}$  and terminal granules. Fig. 18, anaphase I showing bridge. Fig. 19, anaphase II showing 2 lagging chromosomes. Fig. 20, anaphase I showing 6 lagging chromosomes. Fig. 21, metaphase II showing 7 chromosomes at one plate and 5 at other; two exhibit noncongression. Figs. 22-25, *O. rubricapitata* × *O. albinervis*, type II. Fig. 22, diakinesis showing ring of 14 and terminal granules. Fig. 23, anaphase I showing one lagging ring bivalent. Fig. 24, interkinesis showing 2 nucleoli, one at either pole; satellited chromosomes not attached to nucleolus. Fig. 25, anaphase II showing 6 poles.

Varying numbers of free bodies were found inside the nuclear membrane and in some cases in the cytoplasm too, the significance of which is not clear. Figure 5 shows 15 such bodies inside the nucleus and one outside it. These bodies stain with gentian violet in the same way as do the chromosomes.

Varying numbers of granules were found on the threads connecting the ends of the chromosomes at diakinesis. The noncommittal name, terminal granules, suggested by GATES, has been used for them. Since such granules are not present in the somatic chromosomes it is perhaps safe to assume that they may have been



brought about as a result of pairing at pachytene. CATCHESIDE (4) has figured such granules and assumed that they were caused by incomplete terminalization of chiasmata. This explanation is plausible in cases where there is only a single granule between the chromosomes. Sometimes two or three tandem granules (fig. 2) are seen, however, the origin of which is not clear. They may have been caused by the despiralization of the ends of chromosomes, with the result that at diakinesis, when the chromosomes repel each other, the despiralized portions may be relatively more drawn out.

Interlocking of bivalents was often seen. Figure 8 shows one ring bivalent interlocked with a ring of four in *O. biforimiflora cruciata* × *O. angustissima quebecensis*. The same figure shows another bivalent interlocked with a chain of three on one side and one bivalent on the other. The occurrence of interlocking is due to distribution of the threads at zygotene. If two chromosomes pair in such a way that a third chromosome of another pair remains between the pairing threads, chiasma formation at both ends in each pair will bring about interlocking of the bivalents. In *Oenothera* this is a common phenomenon, but it has been observed in other organisms like *Dendrocoelum* (29), *Viviparus* (1), *Allium* (35), and rice (41).

Figure 8 shows 16 chromosomes instead of the usual 14 in *O. biforimiflora cruciata* × *O. angustissima quebecensis* at diakinesis. This higher number may have arisen by the separation of certain daughter chromosomes immediately after their formation by the prophase split during pre-meiotic mitosis, as suggested by GATES (18) in somatic mitosis of *O. lutea*, where he observed cells showing 12, 16, 20, or 21 chromosomes instead of the usual 15. Or it may have arisen owing to double non-disjunction at anaphase of the pre-meiotic mitosis, with the result that both halves of two chromosomes pass to the same pole. If the latter contention should be valid, one naturally expects another pollen mother cell with a lesser number of chromosomes near by. Such a cell was not observed, but this may have been due to the fact that it had been lost during smearing, or it may have lagged in the further developmental stages and got eliminated.

On the assumption that each chromosome consists of three parts (two distal pairing segments homologous with the pairing segments of the adjacent chromosomes in the ring and one median differential region) and that the aggregate of the differential regions accounts for genetic differences between the two gametic complexes, the zigzag arrangement of the ring or chain on the spindle is the usual configuration that will give rise to viable gametes. CATCHESIDE (7), however, points out that there is as yet no evidence in support of the hypothesis that every chromosome has such a differential region.

On the other hand, the failure of this regular zigzag arrangement may lead to the formation of two daughter nuclei with different numbers, one having an extra chromosome and the other lacking one. The result of such nondisjunction is

easily discernible at metaphase II. Figure 13 shows the polar view of metaphase II, with 8 chromosomes at one plate and 6 at the other in *O. eriensis* × *O. Long Island* (species II). Other variations found at this stage are 8 and 5, making a total of 13; and 9 and 6, making a total of 15 in *O. rubricapitata* × *O. albinervis*, in plants devoid of red papillae. This indicates nondisjunction in the last pre-meiotic mitosis. The first recorded observations on nondisjunction were made by GATES (15) in the pollen mother cells of *O. rubrinervis* and by DAVIS (11, 12) in *O. biennis* and *O. lamarckiana*; the significance of these observations in relation to the discovery that *O. lamarckiana* mut. *lata* had 15 somatic chromosomes was soon recognized. FORD (14), in an analysis of nondisjunction in *Oenothera*, concluded that 49 different 8-chromosome gametic types were possible from a ring of 14 chromosomes, on the assumption that the presence of at least one representative of each pairing segment is a necessary condition of gametic viability. He also suggested that nondisjunction increases with increasing number of chromosomes in the ring.

Lagging chromosomes were often observed at anaphase I in nearly all the plants. Figure 23 shows a ring bivalent lagging at this stage in *O. rubricapitata* × *O. albinervis*, plants with red papillae, where the maximum catenation is a ring of 14. Figure 20 shows 6 chromosomes lagging. The spindle in this case is distorted, and such cells may fall behind in the further developmental stages and may be totally eliminated. Figure 9 shows a lagging bivalent, probably due to interstitial chiasma, in *O. biformiflora cruciata* × *O. angustissima quebecensis*. This failure of complete terminalization of the chiasma may be due to arrest because of the change in homology. Similar conditions have been observed before (28) in *O. lata*.

Another interesting feature in *O. rubricapitata* × *O. albinervis* at anaphase I is the presence of a dicentric chromatid which connects the two poles to form a bridge. This may be the consequence of pairing between inverted segments, followed by crossing-over in the relatively inverted regions. The conditions necessitating such pairing have been dealt with (32), so that it is not necessary to cite the various possible configurations here. At pachytene, in favorable material like *Zea* (36), *Chorthippus* and *Stauroderus* (10), and *Tulipa* (47), inversion loops were actually observed, but this was not possible in the present material. At metaphase I the inversions are not usually recognizable or inferred, except when the individual chromosomes remain distinct, as in *Paeonia* (8), *Phalaris* (42), etc. At anaphase I there is no doubt about the occurrence of crossing-over in the relatively inverted segments giving rise to a dicentric chromatid, as just mentioned. This dicentric chromatid stretches across the two poles, forming a bridge (fig. 18). In the figure the bridge has just broken, owing to tension. A dicentric chromosome may also arise through reciprocal translocation between two chromosomes. The fragments left may not be easily visible if they are too small.

Figure 24 shows a pollen mother cell at interkinesis in *O. rubricapitata* × *O. albinervis*. Here two nucleoli are seen, one at each pole, with a chromosome attached to each. The nucleolus at each pole appears to have been organized in connection with the chromosome to which it is seen attached at this stage. The chromosomes show also their dual nature. It is surprising to find that the apparently satellited chromosomes (two at one pole and one at the other) have not organized nucleoli at all. No definite conclusions can be drawn from such few observations, but it has been established in other plants (30, 31) that nucleoli arise usually in connection with the satellited or secondarily constricted chromosomes. It is not clear whether this rule will hold in the case of a complex heterozygote such as *Oenothera*. This problem could not be investigated further, as the material was not treated for such a study. LATTER (34), however, did not observe nucleoli at interkinesis in *Lathyrus*. BHATIA (2) did find nucleoli arising at this stage in wheat.

Figure 21 shows a polar view of metaphase II with 7 chromosomes at one pole and 5 at the other. Two are lost in the cytoplasm. Such chromosomes may develop their own spindles and divide, as will be shown later.

Figure 6 shows a micronucleus at metaphase II in *O. angustissima quebecensis* × *O. angustissima*. Here one plate is seen in side view showing 7 chromosomes, and at a lower focus the polar view of the other metaphase plate shows 6 chromosomes; so it is evident that the micronucleus is formed from a single chromosome. It is not clear at what stage this aberration took place. Probably this micronucleus may have been formed by a single lagging chromosome at anaphase I, which failed to get included in the interkinesis nuclei. If this is correct, then it is not clear why the nuclear membrane did not disappear along with the membranes of the interkinesis nuclei. The chromosome in the micronucleus is still in a resting condition.

Lagging chromosomes were also found frequently at anaphase II in most of the plants (fig. 19). Figure 25 shows the chromosomes grouped at six poles instead of at four in *O. rubricapitata* × *O. albinervis*, plants with red papillae. The extra poles may have been formed by some of the lagging chromosomes developing their own spindle, or it may represent the further developmental stages of a tripolar spindle at anaphase I. Tripolar spindles have been observed in other plants, like rice, in both the first and second divisions (33, 44, 41).

### Discussion

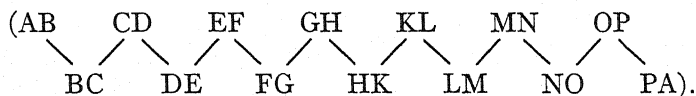
Among the many structural changes characteristic of species evolution, segmental interchange occupies a prominent position, as it has not only a survival value but under certain conditions is specially favored by nature also. Many of the mutations in *Oenothera* depend upon the structural complexity of the chromosomes, which must have arisen from the noncomplex condition through translocations. GATES (24) regards the origin of the linked condition of the chromosomes in

*Oenothera* as due to crossing between different species or types in which one or more pairs of chromosomes are nonhomologous, this condition having arisen by crossing-over or exchange of ends between nonhomologous chromosomes in a species. According to him (21) the ancestral forms of *Oenothera* were homozygous with large flowers, while with decrease in flower size brought about by mutations there was an increase in the catenation of the chromosomes. In *Oenothera* this interchange is maintained in a heterozygous state by a special lethal mechanism associated with homozygosity, while in some other ring-forming plants, like *Rhoeo*, *Campanula*, etc., such mechanisms are only in the process of evolution, as evidenced by the arrangement of the rings.

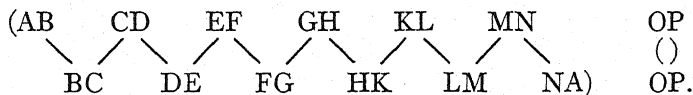
Thus on the assumption that the linked condition of the chromosomes in *Oenothera* arose as a result of successive interchanges in the ancestry of the plant, a plant with the constitution

AB	CD	EF	GH	KL	MN	OP
( )	( )	( )	( )	( )	( )	( )
AB	CD	EF	GH	KL	MN	OP

will by a series of interchanges produce an individual which has a ring of 14 chromosomes having the constitution



On the other hand, segmental interchange between two chromosomes of the same complex in such a plant, like NO and PA, will produce two new chromosomes, NA and OP, so that the resulting plant will have a ring of 12 chromosomes and one bivalent with the constitution



This was inferred from the presence of a lagging ring bivalent (fig. 23) at anaphase I in *O. rubricapitata* × *O. albinervis*, which normally produces a ring of 14 chromosomes in the type with red papillae.

Segmental interchange may take place between homologous or nonhomologous chromosomes. Between homologous chromosomes it is the result of pairing of homologous threads followed by breaks and reunion of the parental threads. But between nonhomologous chromosomes segmental interchange must take place in other ways. According to MORGAN *et al.* (38), translocations may be caused by the

interlocking of nonhomologous chromosomes during the process of synapsis, and CATCHESIDE (5) is of the opinion that this phenomenon may be responsible for segmental interchange. Fragmentation followed by reunion of fragments with broken ends of chromosomes brings about translocation. Apparently all effective translocations are reciprocal where segments are mutually exchanged between two nonhomologous chromosomes (5). Supposed simple translocations (3) have been shown to be reciprocal where one of the segments was very small.

In regard to the mechanism of reciprocal translocation, STADLER (46) considers that it involves random breakage with subsequent reattachment of the broken ends, there being an unlimited interval between the two events. But this is hardly likely, as acentric fragments tend to get lost during mitosis. The most likely view is that of CATCHESIDE (6), who considers that the broken ends join immediately at random when the break occurs at the overlap of two chromosome threads.

DARLINGTON (9) suggests another possibility for the occurrence of segmental interchange between nonhomologous chromosomes. In an organism subject to structural changes in the process of evolution there is the probability of the translocation of an interstitial segment from one chromosome to a corresponding part of one not homologous with it. The steps leading to this involve a series of interchanges. Thus in a chromosome differentiated into three segments, MON, if M and N get interchanged with different segments from two different chromosomes, a new chromosome, POR, may result, leading to the re-duplication of the O segment; and pairing of these two different chromosomes will be the basis for a new interchange—MR and NP chromosomes. In *Pisum*, in a ring of 6 chromosomes (43) crossing-over in the homologous interstitial segment in an otherwise non-homologous chromosome (as just shown) was the cause of a new segmental interchange.

Segmental interchange may be induced by the action of X rays, when its frequency is greatly increased, as in *Drosophila* (13, 39), *Zea mays* (46), etc. NAVA-SHIN and GERASSIMOVA (40) found this phenomenon in seeds germinated after a long period of dormancy. Segmental interchange has been inferred as a result of hybridization in geographical races, as in *Pisum* and *Datura*.

Although segmental interchange between nonhomologous chromosomes occurs in plants like *Pisum* and *Datura*, and a chromosome ring arises as a result of hybridization between the different races involving the interchange, the heterozygous state will not be advantageous to the plant since it produces sterility, owing to the random orientation of the ring on the spindle. In the artificially produced interchange heterozygotes in *Zea*, *Nicotiana*, etc., the same condition results and no further evidence is found of the evolutionary stages leading to the highly evolved complex heterozygote condition as in *Oenothera*.

### Summary

1. Chromosome catenations in *Oenothera* were investigated. The positions of the nucleolar chromosomes in the rings were ascertained in some cases, on the assumption that the particular arrangement is fixed for each plant. In *O. angustissima* × *O. angustissima quebecensis* a pair of satellited chromosomes, which are usually included in the ring of eight, were found attached to the nucleolus at diakinesis.

2. "Terminal granules" were found at either end of the chromosomes at diakinesis, sometimes up to three between two linked chromosomes. When only a single granule was present, apparently it was brought about by failure of the terminalization of chiasmata. The tandem condition of the granules may be due to despi-ralization of the chromosomes at portions near their ends, enhanced by the repulsion between the chromosomes at diakinesis.

3. A dicentric chromatid was observed connecting the two poles at anaphase I in *O. rubricapitata* × *O. albinervis*. Lagging chromosomes were often found at anaphases I and II. Micronuclei were observed in a few pollen mother cells at metaphase II in *O. angustissima quebecensis* × *O. angustissima*, which involved only a single chromosome in one case. They may have been caused by a lagging chromosome at anaphase I or at the anaphase of the pre-meiotic mitosis.

4. In one pollen mother cell at anaphase II in *O. rubricapitata* × *O. albinervis*, plants with red papillae, six poles were observed instead of the usual four. The extra poles may have been due to the lagging chromosomes at first division developing their own spindle, or they may represent the further developmental stages of a tripolar spindle at first division.

5. A lagging ring bivalent was observed at anaphase I in *O. rubricapitata* × *O. albinervis* in the type with red papillae which normally produces a ring of 14 chromosomes. This may be due to segmental interchange between two chromosomes of the same complex. Conditions facilitating segmental interchange are discussed.

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# EFFECTS OF VITAMIN B<sub>1</sub> UPON THE DEVELOPMENT OF SOME FLOWERING PLANTS

CHARLES L. HAMNER

(WITH FOUR FIGURES)

## Introduction

Within the last several years there has been great interest in the possible effects of vitamin B<sub>1</sub> on the growth and development of green plants of economic importance. Although not yet extensive, there is an increasing amount of literature in technical journals (1, 2, 3) and in various publications devoted to horticultural interests. It has been stated that very large increases in the amount of dry weight accumulated occur when small quantities of vitamin B<sub>1</sub> are added to the nutrient medium in which the plants are grown. Such increases are reported as being especially large when the nutrient medium is relatively low in nitrogen content or when short-day plants are grown on short photoperiod as compared with long photoperiod. More briefly it has been stated that vitamin B<sub>1</sub> is effective in promoting increased growth whenever plants are grown under various conditions which are presumed to be suboptimal.

## Material and methods

In the work here reported, some experiments similar to those already published have been performed, and others added. Most of the work was done from March 15 to July 15, 1940, in the greenhouses at the United States Horticultural Station at Beltsville, Maryland, and at the University of Chicago, some of these being repetitions of similar experiments conducted during the previous autumn and winter, with similar end results. Among a number of plants grown, the experiments dealing only with cabbage, cocklebur, cosmos, dahlia, mustard, radish, and zinnia are reported, because similar results were obtained with all varieties of plants used.

The specific treatments are given in detail under the several experiments. For most of the cultures either white quartz sand or washed yellow river sand was used. In all cases a base nutrient composed of 0.0060 mol Ca(NO<sub>3</sub>)<sub>2</sub>, 0.0045 mol MgSO<sub>4</sub>, and 0.0045 mol KH<sub>2</sub>PO<sub>4</sub>, together with a trace of the minor elements, was supplied at least once each week; in many cases it was applied every alternate day. The reaction of the solution was pH 4.8. An amount sufficient thoroughly to saturate the sand in any given container was used at each application. Occasionally the sand was flushed with distilled water. If the plants were grown in soil, tap water was used instead of nutrient solution.

The vitamin B<sub>1</sub> solution was prepared immediately before use, and never stood for more than 2 hours before application. The crystalline preparation made by

Merck and Company was used. After dissolving the specified quantity required for use at any given time in distilled water, the solution was diluted to a concentration of 0.01 mg. in 1 liter of nutrient solution. In the case of the radishes grown in soil, the B<sub>1</sub> was diluted with tap water. Unless otherwise noted, this concentration was employed in all the experiments. At each application of this solution enough was used to saturate the sand in each pot to the degree that a slight amount dripped from the drainage opening. All experiments were set up in such a manner that a statistical analysis of the results could be made. The general plan was to use at least 100 plants in each experiment. They were divided into groups for purposes of randomization. At least eight groups subjected to each type of treatment were used. Many of the plants were grown in small glazed crocks with side delivery openings. All the others, except the radishes, were grown in new porous clay pots which were dipped in a hot paraffin-vaseline mixture to waterproof them before being filled with sand. The containers were placed on slatted frames, raised above the level of the clean bottom of a greenhouse bench, so that none of the drainage water from any container could seep into any other. Similarly, the pots were placed far enough apart that the likelihood of solutions spattering from one to another was eliminated.

Throughout the experiments the weather was somewhat variable, with sunny and cloudy days interspersed. When possible, the greenhouse temperature was maintained at approximately 70° F., but on a few days the outside temperatures ranged somewhat higher, although never in excess of 85° F.

To maintain the conditions of short photoperiod, a framework was constructed over the greenhouse benches, and over this blankets of black sateen cloth were spread to darken the plants for any desired period. The maximum light intensity under such a blanket did not exceed one-half of a foot-candle at any time.

Every effort was made to do the work of harvesting the plants as rapidly as possible. The containers in which the plants were grown were plunged in water, the plants removed with great care, the roots washed in fresh water and then dipped in salt water to aid in removal of the sand. The plants were then blotted, divided into root and top, each sample weighed individually and immediately dried in an electric oven at 80° C. in a rapidly moving current of air. When brittle dry, the plants were weighed again. After drying and weighing, all sand still adhering to the root samples was carefully removed and weighed, and this weight subtracted from the original wet and dry weights, only the final net weights being recorded in the table.

### Experimental results

#### CABBAGE

EXPERIMENT I.—On March 30, 3000 Early Jersey Wakefield cabbage seeds were planted in white quartz sand and watered with nutrient solution. On April 9,

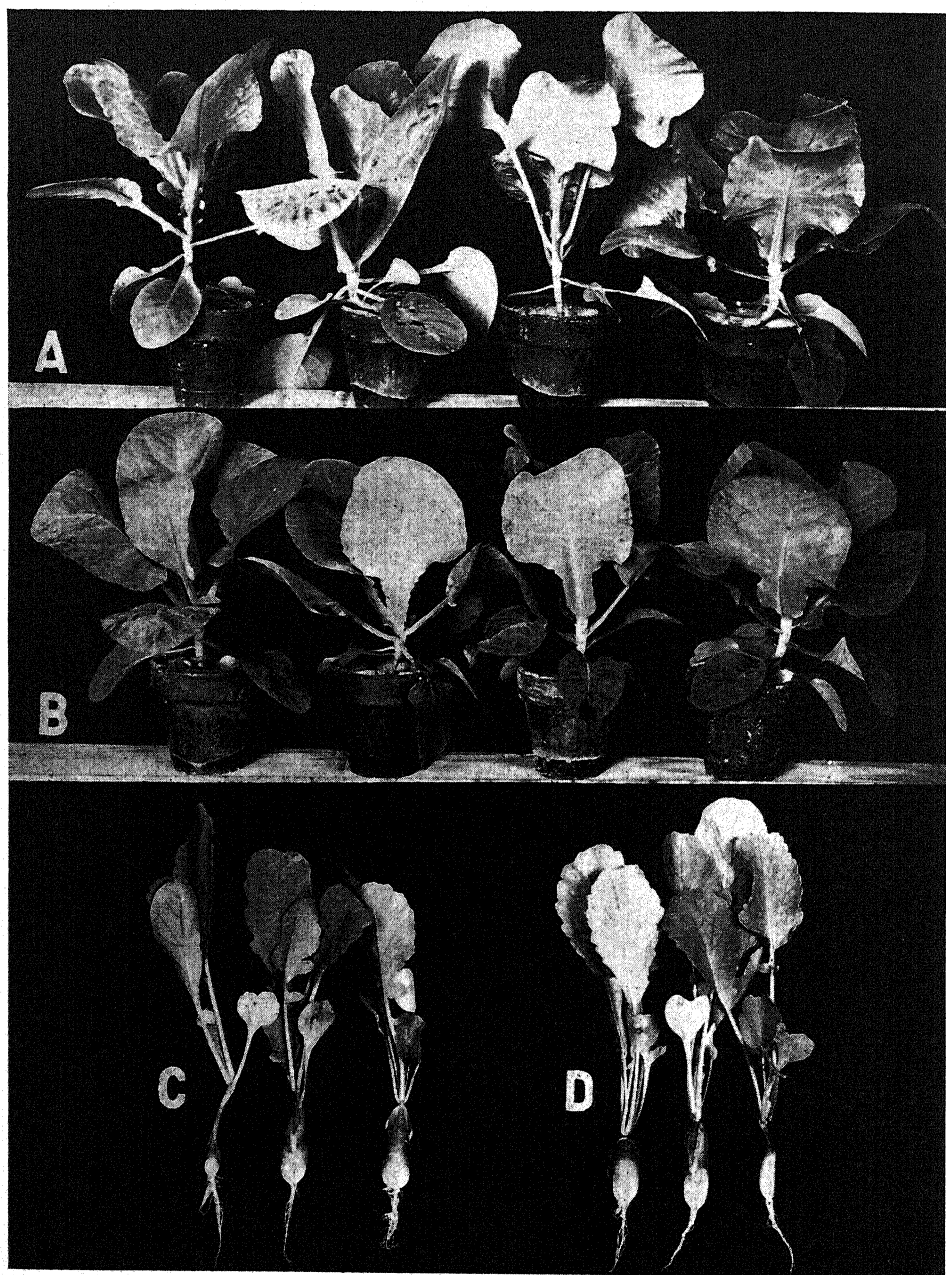


FIG. 1.—Early Jersey Wakefield cabbage (experiment I): *A*, control; *B*, with vitamin B<sub>7</sub> added; *C*, French Breakfast radish (experiment VIII), control; *D*, same with vitamin B<sub>7</sub> added.

seedlings were selected for uniformity and transplanted individually to 4-inch clay pots and placed on a greenhouse bench. All the plants received nutrient solution every day, and in addition half of them received vitamin B<sub>1</sub> once a week. The plants were grown in rows of eight and the treatments were randomized upon the bench. The plants grew very vigorously and were harvested on May 18. No significant differences between those to which B<sub>1</sub> was added and those to which it was not were observed (fig. 1A, B; table 4).

#### COCKLEBUR

Two experiments were carried out, using seeds which had been collected in the autumn of 1939 and stored out of doors during winter. Experiment II was conducted at Beltsville, Md. On March 30, 1200 *Xanthium* seeds were planted in quartz sand. After germination, a number of plants were selected for uniformity and then transplanted. Five plants per crock were used, with a total of thirty-eight crocks. All the plants were placed under a long photoperiod consisting of 17 hours of natural daylight supplemented by 100-watt Mazda lamps out of each 24-hour period. The crocks were supplied with the complete nutrient solution, and half received in addition the vitamin B<sub>1</sub> once each week. Growth was vigorous, although somewhat uneven, and the plants were harvested 47 days after the treatment began (table 4).

Another lot of cocklebur was grown in the greenhouses at the University of Chicago. In this experiment (III) the seeds were planted May 5. Four hundred and ninety plants were grown singly in 1½-liter glazed crocks filled with white quartz sand and watered with nutrient solution. Until June 3 they were maintained under a long photoperiod of 17 hours of natural daylight supplemented by 200-watt Mazda lamps out of each 24-hour period. On May 15 and subsequently, half of the plants were supplied with the B<sub>1</sub> solution twice each week in addition to the nutrient solution. The plants given the two treatments were completely randomized on the benches. On June 3, the photoperiod for half the plants was reduced to 8 hours of natural daylight out of each 24-hour period; the others were continued as previously. The plants were harvested on June 18.

In neither experiment II nor III were significant differences obtained with respect to the vitamin B<sub>1</sub> treatment.

#### COSMOS

Several experiments were conducted. For experiment IV the variety Early Single was used. Under any conditions of culture the plants of this variety show very wide variation in rate of growth, time of blooming, size and color of flowers, and various other characters. Approximately 4000 seeds were planted in quartz sand on March 20 and placed under conditions of long photoperiod, 18 hours

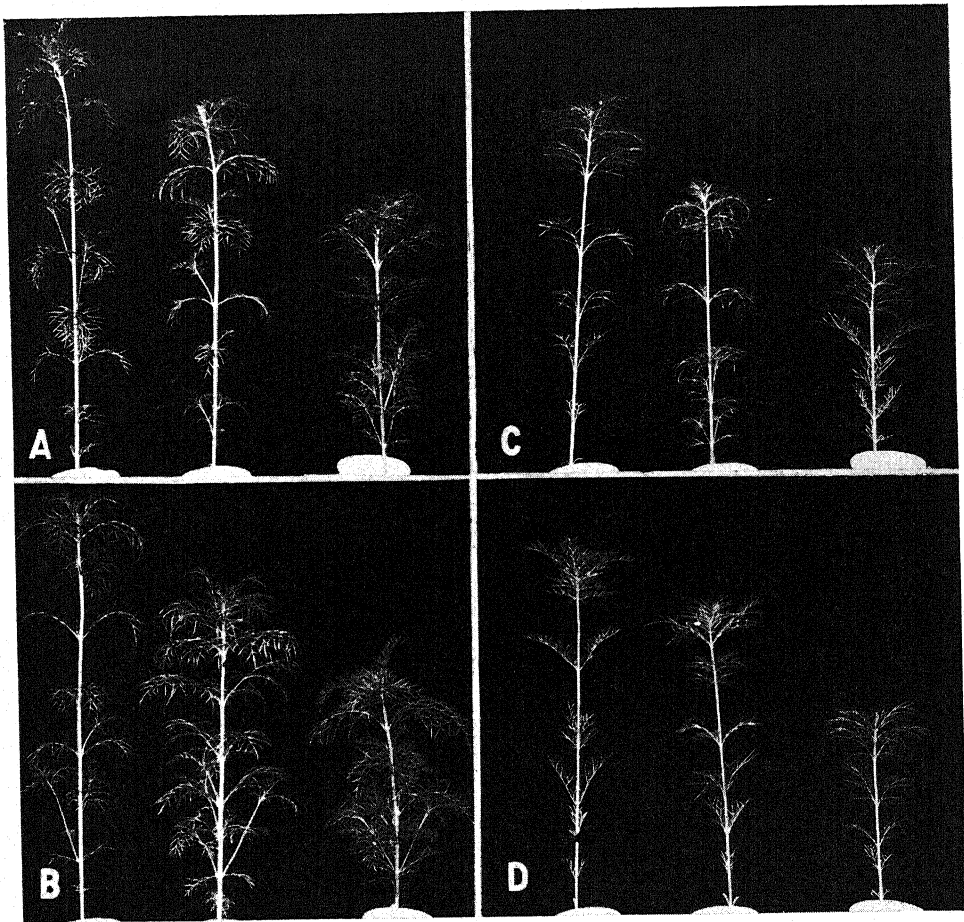


FIG. 2.—Early Single cosmos (experiment IV) grown on long photoperiod, photographed at time of harvest: *A*, grown with complete nutrient solution; *B*, similar to *A* but with vitamin  $B_1$  added; *C*, similar to *A* but with one-fifth the nitrogen in the nutrient solution; *D*, similar to *C* but with  $B_{12}$  added. Plants selected to show range of response to any specific treatment.

of light out of each 24-hour period. When the seedlings were large enough to handle, on March 30, they were selected for uniformity and transplanted—two each to  $1\frac{1}{2}$ -liter glazed crocks filled with quartz sand. Four hundred crocks were placed on each of two benches in the same greenhouse, 800 crocks in all. The plants on one bench were maintained on long photoperiod of 18 hours of daylight supplemented with light from 100-watt Mazda lamps; the other on short photoperiod,  $8\frac{1}{2}$  hours of daylight out of each 24-hour period. The eight series of treatments given on opposite page were established, then divided into lots of ten plants each and completely randomized.

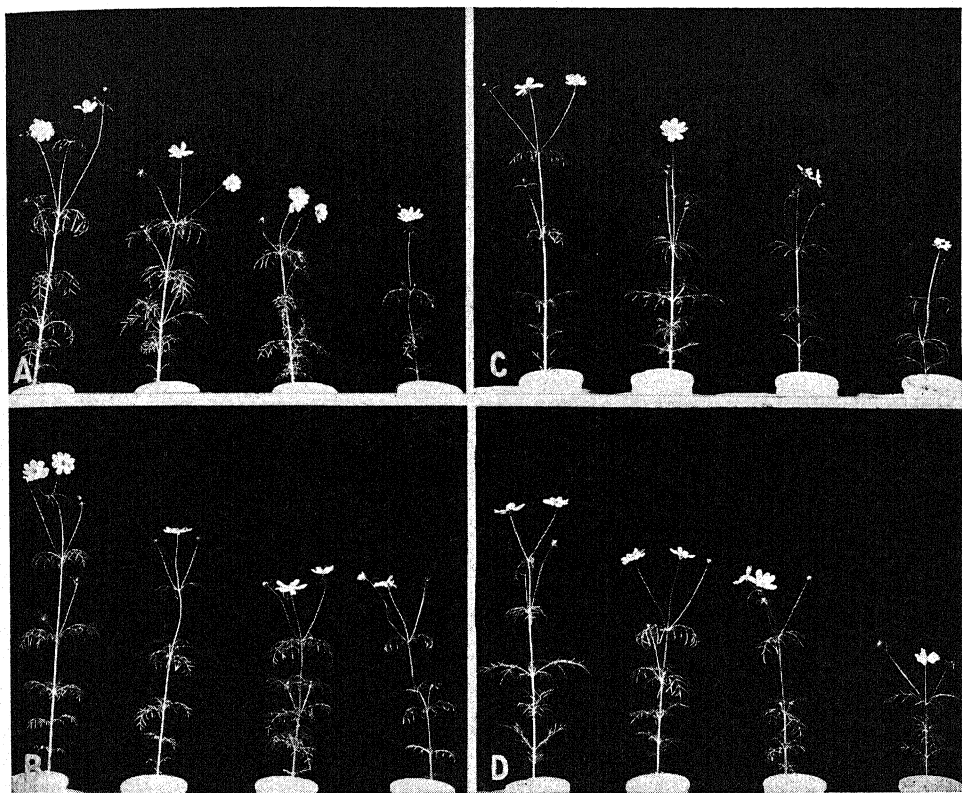


FIG. 3.—Early Single cosmos (experiment IV) grown on short photoperiod: *A*, with complete nutrient solution; *B*, similar to *A* but with vitamin B<sub>1</sub> added; *C*, similar to *A* but with one-fifth as much nitrogen in the nutrient solution; *D*, similar to *C* but with B<sub>1</sub> added. Plants selected on same basis of range of variation as in fig. 2.

1. Long photoperiod, complete nutrient, supplied with vitamin B<sub>1</sub> each week.
2. Similar to 1, but with no B<sub>1</sub> added.
3. Similar to 1, but supplied with nutrient containing one-fifth as much nitrogen.
4. Similar to 3, but with no B<sub>1</sub> added.
5. Short photoperiod, complete nutrient, and supplied with B<sub>1</sub> once each week.
6. Similar to 5, but with no B<sub>1</sub> added.
7. Similar to 5, but supplied with nutrient containing one-fifth as much nitrogen.
8. Similar to 7, but with no B<sub>1</sub> added.

The plants were grown until those on short photoperiod had reached full bloom; they were harvested on May 13. The wet and dry weights of the tops and roots were taken. The plants were harvested using one row of ten plants as a unit; hence for each treatment ten weight determinations were made.

The cosmos plants, although selected for uniformity, showed wide range in growth response under any given treatment (figs. 2 and 3; tables 1, 2).

For experiment V, Burpee Golden cosmos was used. The experiment differed mainly from IV in that four series instead of eight were used, the series on the low plane of nitrogen supply being omitted. Also the short photoperiod was of  $10\frac{1}{2}$  hours' duration instead of  $8\frac{1}{2}$  hours, and the  $B_1$  solution was supplied three times each week instead of once. The seeds were sown May 22, and the plants were harvested July 17 and July 18, at which time all the plants on short photoperiod were in full bloom, as were a few on long photoperiod (table 3).

#### DAHLIA

EXPERIMENT VI.—On April 1, a large quantity of seeds of Coltness Gem dahlia were sown in soil. These germinated promptly, and on April 18, 200 plants were selected for uniformity and transplanted to waxed clay pots containing river sand. On May 17 all the plants were transferred to glazed crocks containing white quartz sand, care being used to disturb the roots as little as possible. All were supplied with nutrient solution; as in the other experiments, half of them received  $B_1$  once each week. The plants grew vigorously and were harvested June 29 (table 4).

#### MUSTARD

EXPERIMENT VII.—On April 30, seedling plants of Giant Southern Curled mustard, germinated in white sand, were selected for uniformity and transplanted singly to waxed clay pots containing white quartz sand. The plants were randomized over a greenhouse bench and watered with complete nutrient solution; one half of them received  $B_1$  once a week. The plants showed uniform growth throughout the experiment, and at no time did any striking differences between the two lots appear. They were harvested May 28 (fig. 4; table 4).

#### RADISH

Three experiments were conducted: two in sand, and one in soil. In experiment VIII, seeds of French Breakfast were sown April 13 directly in wooden flats filled with river sand. After germination, the plants were thinned to approximately forty per flat. Eighteen flats were used, and the treatments were randomized over the bench. All the plants received the full nutrient, and one-half received in addition the  $B_1$  once a week. The plants were harvested May 3 (fig. 1C, D; table 4).

Experiments IX and X were carried on concurrently. Flats were used and randomized as in experiment VIII. For experiment IX the variety Cardinal Globe was used. One-half the seeds were soaked for 2 hours in a 0.01 mg. per liter aqueous solution of vitamin B, and the other half in tap water. The two lots (ten flats of each) were sown separately May 7 in river sand, and the plants thinned as in

TABLE 1

DRY WEIGHT IN GRAMS OF EARLY SINGLE COSMOS PLANTS GROWING UNDER SHORT PHOTOPERIOD, WITH AND WITHOUT VITAMIN B<sub>1</sub> AND WITH COMPLETE NUTRIENT SOLUTION, AT HIGH AND LOW PLANES OF NITROGEN SUPPLY. EACH OF THE FOUR LOTS (WITH AND WITHOUT VITAMIN B<sub>1</sub>) WAS RANDOMIZED ON THE BASIS OF 10 SAMPLES, EACH CONSISTING OF 10 PLANTS

	HIGH NITROGEN				LOW NITROGEN			
	WITH B <sub>1</sub>		WITHOUT B <sub>1</sub>		WITH B <sub>1</sub>		WITHOUT B <sub>1</sub>	
	TOPS	ROOTS	TOPS	ROOTS	TOPS	ROOTS	TOPS	ROOTS
	10.7	2.0	11.0	1.3	7.3	1.1	7.6	1.1
	11.1	1.6	11.7	1.6	7.8	1.2	7.7	1.5
	8.0	1.2	10.3	2.3	6.3	0.8	8.9	1.5
	8.7	1.1	9.2	1.1	7.4	1.3	7.0	1.0
	10.6	1.1	9.0	1.1	8.3	1.1	7.5	1.0
	9.2	1.1	9.6	1.3	7.6	1.0	7.6	1.4
	8.3	1.3	10.4	1.5	7.2	1.0	7.4	1.3
	11.9	1.4	10.1	1.0	7.0	1.1	8.8	1.5
	11.4	1.7	11.0	1.3	9.3	1.3	7.7	1.4
	11.1	1.9	11.2	1.4	7.3	1.1	7.7	1.4
Average:								
Per 10 plants.....	10.1	1.44	10.35	1.39	7.55	1.10	7.79	1.31
Per plant.....	1.01	0.13	1.03	0.139	0.75	0.110	0.779	0.131

TABLE 2

DRY WEIGHT IN GRAMS OF EARLY SINGLE COSMOS PLANTS GROWING UNDER LONG PHOTOPERIOD, WITH AND WITHOUT VITAMIN B<sub>1</sub> AND WITH COMPLETE NUTRIENT SOLUTION, AT HIGH AND LOW PLANES OF NITROGEN SUPPLY. EACH OF THE FOUR LOTS (WITH AND WITHOUT VITAMIN B<sub>1</sub>) WAS RANDOMIZED ON THE BASIS OF 10 SAMPLES, EACH CONSISTING OF 10 PLANTS

	HIGH NITROGEN				LOW NITROGEN			
	WITH B <sub>1</sub>		WITHOUT B <sub>1</sub>		WITH B <sub>1</sub>		WITHOUT B <sub>1</sub>	
	TOPS	ROOTS	TOPS	ROOTS	TOPS	ROOTS	TOPS	ROOTS
	41.0	10.6	40.0	13.9	17.2	5.6	17.9	7.1
	35.0	16.7	32.5	11.5	18.2	6.4	16.9	5.9
	42.5	11.9	36.9	9.8	19.6	6.2	19.4	6.1
	39.7	10.0	37.8	10.8	17.6	6.6	15.8	5.9
	40.5	11.0	38.3	10.6	16.9	5.8	16.8	6.6
	40.3	10.6	43.1	11.1	16.9	5.7	14.4	3.7
	43.5	11.4	38.5	9.0	16.5	5.8	17.2	5.9
	43.1	10.5	39.8	10.5	19.1	5.9	18.0	4.9
	40.6	9.4	42.5	11.6	19.5	6.1	18.0	5.6
	41.3	10.2	36.3	8.3	17.9	5.1	16.4	4.6
Average:								
Per 10 plants.....	40.75	11.23	38.57	10.71	17.94	5.92	17.08	5.63
Per plant.....	4.07	1.12	3.85	1.07	1.79	0.59	1.70	0.56



experiment VIII. All were watered with full strength nutrient solution and with tap water. The lot of plants from the seeds soaked in  $B_1$  solution received in addition the  $B_1$  once a week. The plants were harvested June 3.

For experiment X the variety Crimson Giant was used. The seeds were soaked in the same way as in experiment IX, but they were sown in flats of garden loam instead of river sand, on May 7. Two lots of nine flats each were randomized as usual, and watered with tap water. To the plants from the seeds steeped in  $B_1$

TABLE 3

DRY WEIGHT IN GRAMS OF BURPEE GOLDEN COSMOS PLANTS GROWING UNDER LONG PHOTOPERIOD, WITH AND WITHOUT VITAMIN  $B_1$  AND SHORT PHOTOPERIOD WITH COMPLETE NUTRIENT. EACH OF THE FOUR LOTS (WITH AND WITHOUT VITAMIN  $B_1$ ) WAS RANDOMIZED ON THE BASIS OF 11 SAMPLES, EACH CONSISTING OF 10 PLANTS

	LONG PHOTOPERIOD				SHORT PHOTOPERIOD			
	WITH $B_1$		WITHOUT $B_1$		WITH $B_1$		WITHOUT $B_1$	
	TOPS	ROOTS	TOPS	ROOTS	TOPS	ROOTS	TOPS	ROOTS
	47.0	9.9	48.0	8.5	26.5	3.2	25.5	3.0
	49.5	9.6	50.0	11.1	39.5	4.8	41.5	5.8
	51.5	9.3	55.5	10.1	35.5	5.0	39.0	6.4
	44.0	9.8	48.5	9.9	36.0	4.9	28.0	3.9
	46.0	11.2	66.0	12.0	43.5	5.3	39.0	5.7
	52.5	9.7	47.0	9.5	41.5	6.2	35.0	5.0
	50.5	11.7	53.0	10.6	36.5	6.1	40.0	6.0
	53.0	10.8	57.5	12.4	36.5	5.3	40.5	5.6
	53.5	9.6	48.5	12.3	43.0	5.9	40.0	5.7
	47.5	9.2	51.5	10.5	40.5	5.7	39.0	4.9
	46.0	8.0	73.5	13.2	34.0	5.0	42.0	6.1
Total.....	547.0	108.8	599.0	120.1	413.0	57.4	409.5	58.1
Average								
Per 10 plants.....	49.72	9.9	54.45	10.91	37.4	5.21	37.22	5.28
Per plant.....	4.97	0.99	5.45	1.09	3.74	0.521	3.72	0.528

solution the usual  $B_1$  solution was added once each week; the others received tap water only. The plants were harvested June 3.

In all the experiments on radish no significant differences were observed (table 4).

### ZINNIA

EXPERIMENT XI.—On April 10, 2000 seeds of Star Dust variety of zinnia were planted in quartz sand. After germination, plants were re-selected for uniformity and on April 22 about 500 were transplanted singly into 4-inch waxed clay pots. Two hundred and twenty-four were again selected for uniformity and randomized on a greenhouse bench in rows of eight. All the plants were watered with nutrient

TABLE 4  
EXPERIMENTS ON SEVERAL VARIETIES OF PLANTS, SPECIFIC TREATMENTS  
AS INDICATED; WEIGHT IN GRAMS

VARIETY OF PLANT	No. OF EXPERI- MENT	SPECIFIC TREATMENT	No. OF PLANTS USED	No. OF RANDOM SAMPLES	AVERAGE DRY WEIGHT PER PLANT (GM.)	
					Tops	Roots
Cabbage (Early Jersey Wakefield).....	I	Grown in sand with complete nutrient: B <sub>1</sub> added.....	104	13	5.36	1.23
		Control*.....	104	13	5.29	1.30
Cocklebur.....	II	In sand with complete nutrient on long photoperiod: B <sub>1</sub> added.....	95	19	4.24	1.11
		Control.....	95	19	4.18	1.09
Cocklebur.....	III	In sand with complete nutrient on long photoperiod: B <sub>1</sub> added.....	180	18	3.68	0.90
		Control.....	180	18	3.64	0.92
		Transferred to short photo-period for 2 weeks just prior to harvesting B <sub>1</sub> added.....	200	20	3.85	0.72
		Control.....	200	20	3.81	0.72
Cosmos (Early Single) ..	IV	(See tables 1 and 2)				
Cosmos (Burpee Golden)	V	(See table 3)				
Dahlia (Coltness Gem) ..	VI	In sand with complete nutrient solution: B <sub>1</sub> added.....	91	13	6.36	3.41
		Control.....	91	13	6.46	3.10
Mustard (Giant Southern Curled).....	VII	In sand with complete nutrient solution: B <sub>1</sub> added.....	88	11	2.67	0.53
		Control.....	88	11	2.68	0.53
Radish (French Break-fast).....	VIII	In sand with complete nutrient solution: B <sub>1</sub> added.....	432	9	0.205	0.153
		Control.....	432	9	0.210	0.156
Radish (Cardinal Globe)	IX	In sand with complete nutrient solution: B <sub>1</sub> added.....	320	8	0.289	0.107
		Control.....	320	8	0.313	0.105
Radish (Crimson Giant)	X	In soil: B <sub>1</sub> added.....	312	8	0.478	0.232
		Control.....	312	8	0.508	0.233
Zinnia (Star Dust).....	XI	In sand with complete nutrient solution: B <sub>1</sub> added.....	112	14	1.96	0.38
		Control.....	112	14	1.95	0.38

\* No. B<sub>1</sub> added to culture solution or soil.

solution, and one-half of them were given in addition the  $B_1$  solution once each week. All the plants grew vigorously but showed considerable range of variation, irrespective of treatment given. They were harvested on June 3 (table 4).



FIG. 4.—Giant Southern Curled mustard (experiment VII): *A*, control; *B*, with vitamin  $B_1$  added. Photographed at time of harvest.

### Discussion

At the present time it is not possible to account for the discrepancies between the results of the experiments reported here and those of BONNER and GREENE (2, 3) and of BONNER (1). The individual plants in the present experiments were many times larger and very much heavier than those reported by them. For example, the average weight per plant of the tops in grams of cosmos plants grown by BONNER and GREENE under long photoperiod for a period of 8 weeks was 0.0675

gm. for the controls and 0.110 gm. for the plants to which B<sub>1</sub> had been added. In the present experiment with Early Single cosmos, the average weight per plant under long photoperiod for 6 weeks was 3.85 gm. for the controls and 4.07 gm. for those to which B<sub>1</sub> had been added in the culture medium. In the case of Burpee Golden cosmos the differences were still greater. Similarly, in experiments on cocklebur grown on long photoperiod for 4 weeks and which were 7 weeks old when harvested (1), the weight of the tops of control plants averaged 0.810 gm. and those to which B<sub>1</sub> had been added, 1.00 gm. In the experiments reported here, the average weight per plant of cocklebur grown under similar conditions of photoperiod was four times as great. Little difference was shown between the controls and those to which B<sub>1</sub> had been added.

As already stated, the pH of the nutrient solution was 4.8; alkaline conditions never prevailed throughout the entire length of any experiment. The solutions of vitamin B<sub>1</sub> were always prepared immediately before use.

In the cases of Early Single cosmos, cocklebur, zinnia, and radish the range of variation among the plants of the several varieties was large. It is certainly true, as the detailed figures and the illustrations clearly show, that variation within experimental lots was often as great under any given set of conditions imposed as between different experimental conditions. Because of this fact, large populations, thoroughly randomized, were always used. In the case of cabbage and mustard, the plants were strikingly uniform in appearance in any given lot and showed little difference between controls and those to which B<sub>1</sub> had been added.

To say that the addition of vitamin B<sub>1</sub> is of particular value in increasing growth or dry weight accumulation when added to plants of any of the varieties or species tried—when they are subjected to growth limitation through photoperiod or nutrient supply—is not in keeping with the results of these experiments. There is marked difference between the lots of plants grown on long photoperiod as contrasted with those grown on short photoperiod, on a high plane of nitrogen supply as compared with those on a low one; but the addition of vitamin B<sub>1</sub> in no way changed this relationship. Statistically the differences which could be ascribed to the addition of B<sub>1</sub> as compared with a control under similar environment were never significant.

### Summary

1. Vitamin B<sub>1</sub> was added to a number of different plants in a concentration of 0.01 mg. per liter. In most of the experiments the plants received vitamin B<sub>1</sub> in the nutrient solution once a week; however, one of the cocklebur experiments received vitamin B<sub>1</sub> twice a week, and in one of the cosmos experiments vitamin B<sub>1</sub> was added three times a week.

2. No visible differences were detected between the control plants and those re-

ceiving B<sub>1</sub>. No significant difference was obtained in wet weight or in the accumulation of dry matter.

3. Under neither a long nor a short photoperiod, nor at high nor low planes of nitrogen nutrition, were there detectable differences which could be ascribed to additions of vitamin B<sub>1</sub> to the cultures.

4. Vitamin B<sub>1</sub> had no effect upon hastening flowering, upon size or number of flowers produced, or upon color or quality of the flowers of cosmos.

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#### LITERATURE CITED

1. BONNER, JAMES, Experiments on photoperiod in relation to the vegetative growth of plants. *Plant Physiol.* 15:319-326. 1940.
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# RESPONSES OF MARCHANTIA POLYMORPHA TO NUTRIENT SUPPLY AND PHOTOPERIOD<sup>1, 2</sup>

CONTRIBUTIONS FROM THE HULL BOTANICAL LABORATORY 515

PAUL D. VOTH AND KARL C. HAMNER

(WITH FOURTEEN FIGURES)

## Introduction

As experimental material in studies on plant nutrition, *Marchantia polymorpha* L. has been used for many years. Various substrates have been used, including soil (5, 29, 31, 33), agar with various nutrients (6, 8, 10, 23, 34), blotting or filter paper (5, 6, 29), sand (29), cinders (38), peat (29, 31), and nutrient solutions (1, 5, 9, 10, 39). These investigations have utilized gemmae in the main and include studies of dorsiventrality (1, 5, 8, 29, 31, 39), effect of the concentration and composition of nutrient solution (1, 2, 10, 11, 23, 33), effect of temperature variations (1, 5, 9, 10, 11), intensity and quality of light (5, 6, 8, 9, 10, 11, 23, 34, 39), and photoperiod (5, 38). Work on seed plants (17, 28) has indicated a possible inter-relationship between mineral nutrition and photoperiod. The present studies deal principally with similar relationships in *Marchantia*, with particular reference to the development of the gametophyte and gametangiophores.

*Marchantia* grows readily on moist soil, but our attempts to grow it in sand culture have been more or less unsuccessful because of the tendency of the surface of the sand to become dry, and consequently the plants do not obtain sufficient moisture. When a system of continuous irrigation in sand was used, salts tended to accumulate on the surface of the sand, resulting in injury to the plants. Most of these difficulties were avoided by the use of methods subsequently described in this paper. Plants were grown on a wide variety of nutrient solutions, under various conditions of photoperiod. Records were made in each case of changes in the gross appearance of the plants, increase in total area, formation of gemmae cups and gametangiophores, anatomical variations, and the accumulation of dry weight.

## General methods

The strains of *Marchantia polymorpha* used in these experiments produce (as usually grown) antheridiophores in late February and early March and arche-goniophores a few weeks later. Gemmae cups are produced abundantly in autumn

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and winter. The experiments described here were conducted in the greenhouses at the University of Chicago during the late spring, summer, and fall of 1939 and the spring of 1940.

Sand culture having proved unsatisfactory, floats of balsa wood were devised to support the thallus and to keep it in contact with the surface of the culture solu-

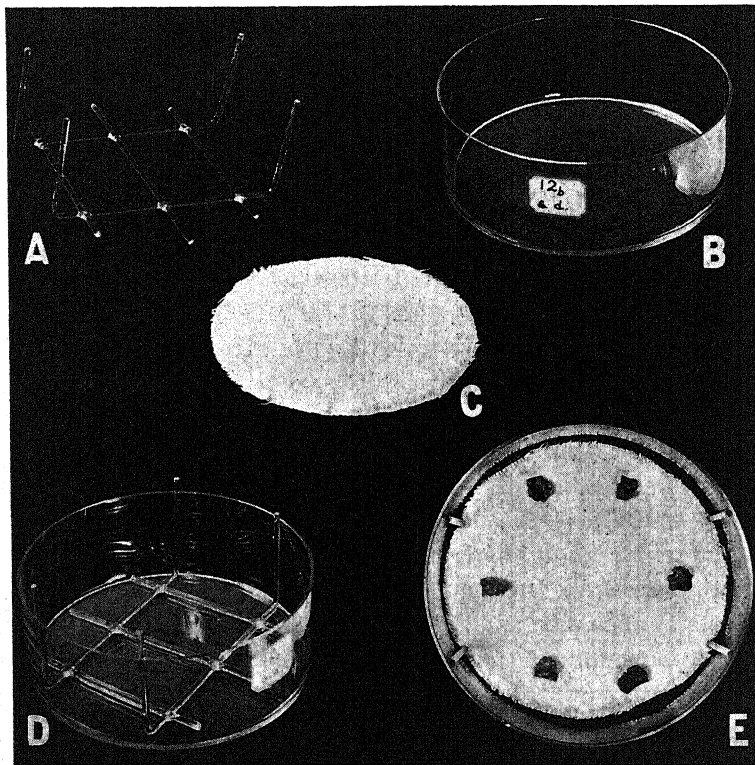


FIG. 1.—Apparatus used in growing *Marchantia*: A, glass rack or framework welded from pieces of glass rod, 5 mm. in diameter; B, half a glass moist chamber, approximately 9×3 inches; C, glass cloth; D, glass rack in moist chamber; E, complete assembly with six cuttings.

tion. This method was abandoned because the floats tended to become water logged, even though impregnated with paraffin.

After several preliminary trials, it was found that *Marchantia* would grow well if placed upon a coarse grade of glass cloth in contact with nutrient solution. The cloth served as a wick and was moist throughout its area if kept in contact with the solution at one or two points. The setup used is illustrated in figure 1. Half of a moist chamber was placed on a greenhouse bench with the open face upward. Glass rods were welded together to form a circular framework which would just fit into the chamber, and over this framework a circular piece of glass cloth was so placed that it was approximately  $\frac{1}{4}$  inch above the bottom of the chamber.

Sufficient culture solution was added to come into contact with the lower surface of the cloth. Extensions of the two longer rods of the framework were bent upward to serve as handles in facilitating removal from the moist chamber. Before use in establishing a culture in any experiment, the entire setup was thoroughly cleansed by treatment for 24 hours in cleaning solution and then thoroughly washed in tap water, followed by distilled water. All nutrient solutions were changed every day or every other day, depending upon the temperature and the amount of evaporation. Every second day the framework and glass cloth were removed and rinsed thoroughly in distilled water, and fresh nutrient solution was again placed immediately in the moist chamber.

Using this method, an attempt was made to grow *Marchantia* on a solution which had an osmotic concentration of approximately 0.79 atmospheres. At full strength this solution consisted of 12 cc. of 0.5M  $\text{Ca}(\text{NO}_3)_2$ , 9 cc. of 0.5M  $\text{KH}_2\text{PO}_4$ , 9 cc. of 0.5M  $\text{MgSO}_4$ , and 1 p.p.m. of some of the microelements—except  $\text{FeSO}_4$ , which was supplied at 0.1 p.p.m. (16). The plants grew fairly well in this solution when compared with similar plants grown on soil. There was, however, some evidence of salt excess in the nutrient cultures; consequently, several experiments were conducted in which the growth of *Marchantia* on the full strength solution was compared with its growth on dilutions of one-fifth and one-tenth the original concentration. Control plants were grown on soil, tap water, and distilled water. Responses to the one-tenth dilution were similar to the one-fifth dilution. Because of the growth responses, production of gametangiophores and gemmae cups, and area of thallus developed, the concentration of solution used as a basis in making up the nutrient combinations in all subsequent experiments was comparable to that of the one-fifth dilution of the solution originally employed. It is not known whether the greater growth was owing to a lower total osmotic concentration or to a lower concentration of certain of the individual inorganic salts. In all subsequent work the solutions had an osmotic concentration of approximately 0.285 atmospheres.

A range of nutrient solutions was used according to the method reported by C. L. HAMNER (16). Six stock solutions were prepared with molal concentrations as given in table 1. Each of three of these contained only K, Ca, or Mg as the cation, but all contained the three anions  $\text{NO}_3$ ,  $\text{PO}_4$ , or  $\text{SO}_4$ . Each of the other three contained only  $\text{NO}_3$ ,  $\text{PO}_4$ , or  $\text{SO}_4$  as the anion, but had the cations K, Ca, and Mg. This was accomplished by the use of nine salts, as indicated in table 1. By the use of these solutions, and by effecting various combinations of them on the basis of sixths, it was possible to arrange a triangle of twenty-eight different solutions with special reference to the cations present and an additional twenty-eight with particular reference to the anions. The combinations composing each triangle were placed in order on the basis of their relative composition and



numbered (fig. 2). The twenty cultures on short photoperiod were supplied with solutions such as occurred only in the central portion of the triangle, ten representing the cations and ten the anions. The pH of all stock solutions (table 1) and those supplied the plants (fig. 2) was between 5.0 and 6.0. All solutions became neutral or slightly alkaline after being in the moist chambers in contact with the growing plants for a day, except the solutions lacking in nitrogen, which changed very little.

The conditions for long photoperiod were obtained by means of 200-watt Mazda lamps with reflectors suspended about 3 feet above the greenhouse bench. These lamps burned from sundown until 2:00 A.M. each night, supplying a maximum illumination of approximately 100 foot-candles at the surface of the plant.

TABLE 1

CONCENTRATIONS OF SALTS IN THE 6 STOCK NUTRIENT SOLUTIONS USED IN MAKING UP 56 OTHER COMBINATIONS. TO EACH STOCK SOLUTION WAS ADDED 0.2 P.P.M. OF  $\text{MnSO}_4$ ,  $\text{ZnCl}_2$ , AND  $\text{Na}_2\text{B}_4\text{O}_7$ , AND ALSO 0.02 P.P.M. OF  $\text{FeSO}_4$

SALT	MOLAL CONCENTRATIONS OF INDIVIDUAL STOCK SOLUTIONS					
	K	Ca	Mg	$\text{NO}_3$	$\text{PO}_4$	$\text{SO}_4$
$\text{KNO}_3$ .....	0.0024	.....	.....	0.0009	.....	.....
$\text{KH}_2\text{PO}_4$ .....	0.0009	.....	.....	.....	0.0009	.....
$\text{K}_2\text{SO}_4$ .....	0.0009	.....	.....	.....	.....	0.00045
$\text{Ca}(\text{NO}_3)_2$ .....	.....	0.0012	.....	0.0012	.....	.....
$\text{Ca}(\text{H}_2\text{PO}_4)_2$ .....	.....	0.00045	.....	.....	0.0012	.....
$\text{CaSO}_4$ .....	.....	0.0009	.....	.....	.....	0.0012
$\text{Mg}(\text{NO}_3)_2$ .....	.....	.....	0.0012	0.0009	.....	.....
$\text{MgHPO}_4$ .....	.....	.....	0.0009	.....	0.0009	.....
$\text{MgSO}_4$ .....	.....	.....	0.0009	.....	.....	0.0009

The short photoperiod was obtained by building a framework over the bench and covering it completely with black cloth at 5:00 P.M. every evening. The black cloth was removed at 8:00 A.M. each morning. Thus the plants exposed to long photoperiod received approximately 18-20 hours of light out of each 24-hour period, and plants exposed to short photoperiod received 9 hours of light.

All the plants were propagated vegetatively from a single male plant and a female plant. They were grown in the greenhouse on a one-fifth dilution of a three salt solution (16) and furnished the stock from which the plants used for experimentation were taken. At the beginning of any particular experiment, a small portion from the tip of a branch of the stock was taken. Six of these cuttings were selected for uniformity and arranged equidistantly on the glass cloth (fig. 1E). The growing point of each cutting was directed toward the center of the dish. At the time of making the cuttings, the total area of each particular group of six was determined by means of an area photometer (24). Several representative

groups of six cuttings designated as initial controls were also set aside for fresh and dry weight determinations, and other representative cuttings were fixed for subsequent sectioning in a formalin-acetic-alcohol solution consisting of 67 cc. forma-

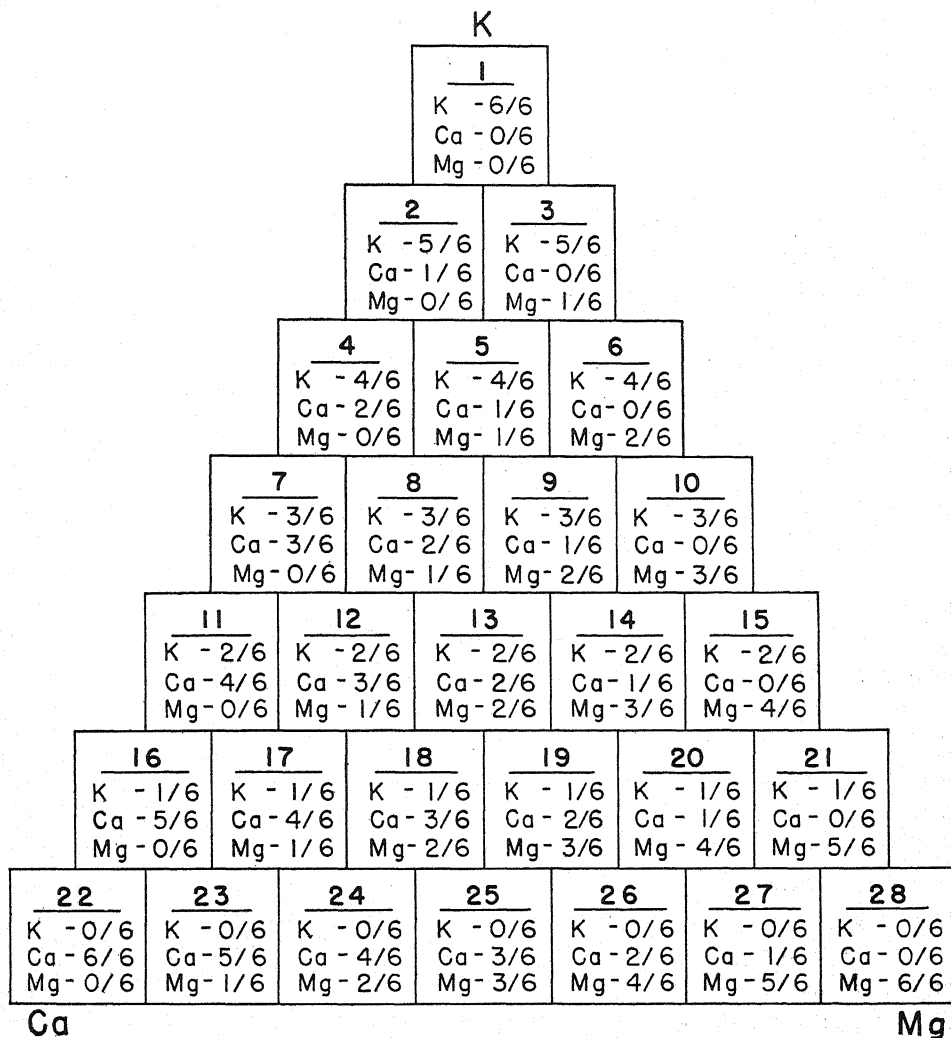


FIG. 2.—Triangle showing method of combination of stock solutions on the basis of sixths, to secure a range of 28 solutions of varying cations. Each apical position lacks two cations; each side of the triangle lacks one; and the center is supplied with all three in varying proportions. A triangle for varying the combination of anions could be effected in a similar manner.

lin, 23 cc. glacial acetic acid, 500 cc. of 95 per cent alcohol, and water sufficient to make 1 liter.

At the completion of an experiment, the plants were carefully loosened from the glass cloth by means of a sharp spatula, each group was photographed, and the

total area was determined. Careful notes were made as to the number of gemmae cups, the number of antheridiophores or archegoniophores, and the general appearance of the plants. The fresh weight of the plants was then determined, and one plant selected for fixation in F.A.A. and subsequent sectioning. The fresh weight of the remaining five plants was determined. They were then dried at 102° C. On the basis of the percentage dry weight of five plants, and the fresh weight of the six plants, the total dry weight of six plants was calculated.

### Investigation

#### DETAILS OF SPECIFIC EXPERIMENTS

EXPERIMENT 8.—On March 14, 1940, fifty-six cultures were placed under conditions of long photoperiod and twenty under short photoperiod. The six cuttings in each culture totaled 15 sq. cm. in area. In any set of six plants there were approximately 16-18 growing points. Four more cultures served as initial controls. The fresh and dry weights of each lot varied very little. The average dry weight was 60 mg. The character of growth, development, and final results obtained under conditions of short photoperiod are described later in a special section. The growth and general appearance of the plants subjected to conditions of long photoperiod at progressive dates were as follows.

On March 22, 8 days after beginning the experiment, progressive death was noted in certain of the cultures in the cation triangle on long photoperiod. The growing points of the plants supplied with nutrient solutions lacking in calcium were dead, and necrosis in the wings of the thallus followed. The most rapid development of this symptom occurred in nutrient solution 28. The other solutions lacking calcium ranged in this order of toxicity: 21, 15, 10, 6, 3, and 1. While all these solutions are lacking in calcium, they contain various proportions of magnesium and potassium. Plants on the solutions relatively high in magnesium and low in potassium seemed to develop the calcium deficiency symptoms more rapidly than did those on solutions high in potassium and low in magnesium. The first evidence of calcium deficiency was darkening of the growing point, which later became translucent, then very black, and finally died completely. Fourteen days after the start of the experiment, or March 28, the calcium deficiency symptoms were much more apparent. On April 2, growing points of all the plants supplied with a nutrient solution lacking calcium were dead, and portions of the thallus were disintegrated. On April 16 there was some regeneration in the plants growing on solutions lacking in calcium. This was particularly true with the plants on solutions 1, 3, 6, and 10. Slight regeneration was apparent in the plants on solution 15, and those plants on solutions 21 and 28 were apparently dead.

There were no particular symptoms of magnesium deficiency on April 2, except that the plants on solutions lacking magnesium grew less than some of the others.

The greatest growth in the cation triangle was made by plants receiving solutions 8, 11, 12, 13, 17, and 18. The plants supplied with nutrient solutions lacking potassium did not grow so well, and the older portions of the thallus of such plants had died. This portion of the thallus was often straw colored rather than bright green. On April 16, approximately 50 per cent of the base of each plant supplied with solutions lacking in potassium was dead. There was very little evidence of magnesium deficiency in any of the plants at this time, except perhaps that the plants on solutions lacking magnesium did not grow quite so rapidly. No evidence of potassium or calcium excess symptoms was recorded. There may have been some effect of excess in magnesium, as evidenced by the fact that plants on solution 28 were apparently dead early in the course of the experiment. Plants on solutions 21 and 15 also appeared dead by the time of harvest. The fact that solutions 21 and 15 were not so favorable for growth as were solutions 1 and 3 may have been owing to the presence of a higher concentration of magnesium in solutions 21 and 28 than in solutions 1 and 3, or possibly to the lower concentration of potassium in the former as compared with the latter. All these solutions, of course, contained no calcium.

By March 28, certain types of growth responses were evident in the plants of the anion triangle exposed to long photoperiod. Those plants growing on solutions lacking phosphorus were somewhat greener than the others, and certain of them developed a green-black color in the midrib region. By April 2, all the plants on nutrients lacking phosphorus had black midribs. At this time the greatest growth in the anion triangle was on solutions 31, 33, 34, 36, 37, and 38. Up to this time absence of phosphorus had not resulted in appreciable decrease in the rate of growth, if at all. On April 16, the plants on solutions lacking phosphorus all exhibited extensive growth and were of darker green color than plants on any of the other solutions. Particularly the regions near the tips of the thallus were dark green, the midrib was black or very deep red, and the bases of the plants were tan. On this date, those plants growing on solutions lacking nitrate were stunted, producing much less growth than those on other solutions. They had a red coloration which at first was ascribed to anthocyanin in the scales on the ventral surface of the thallus. This red color and the color of the plants lacking in phosphorus will be discussed in the anatomical section. The margins of the thalli possessed very little chlorophyll and were almost white. Those plants growing on solutions lacking in sulphate produced slightly less growth than those on some of the other solutions. There was some evidence of phosphate excess in plants growing on solutions 50, 44, 51, 39, 45, and 52. The margins of these plants were necrotic and subsequently tended to dry up, as if burned. This symptom, however, was not very marked at any time.

All plants were harvested on April 16 (figs. 3, 4, 7, 8). The records of the num-

EXP. 8  
8 PLANTS

K

INITIAL CONTROL

INITIAL CONTROL

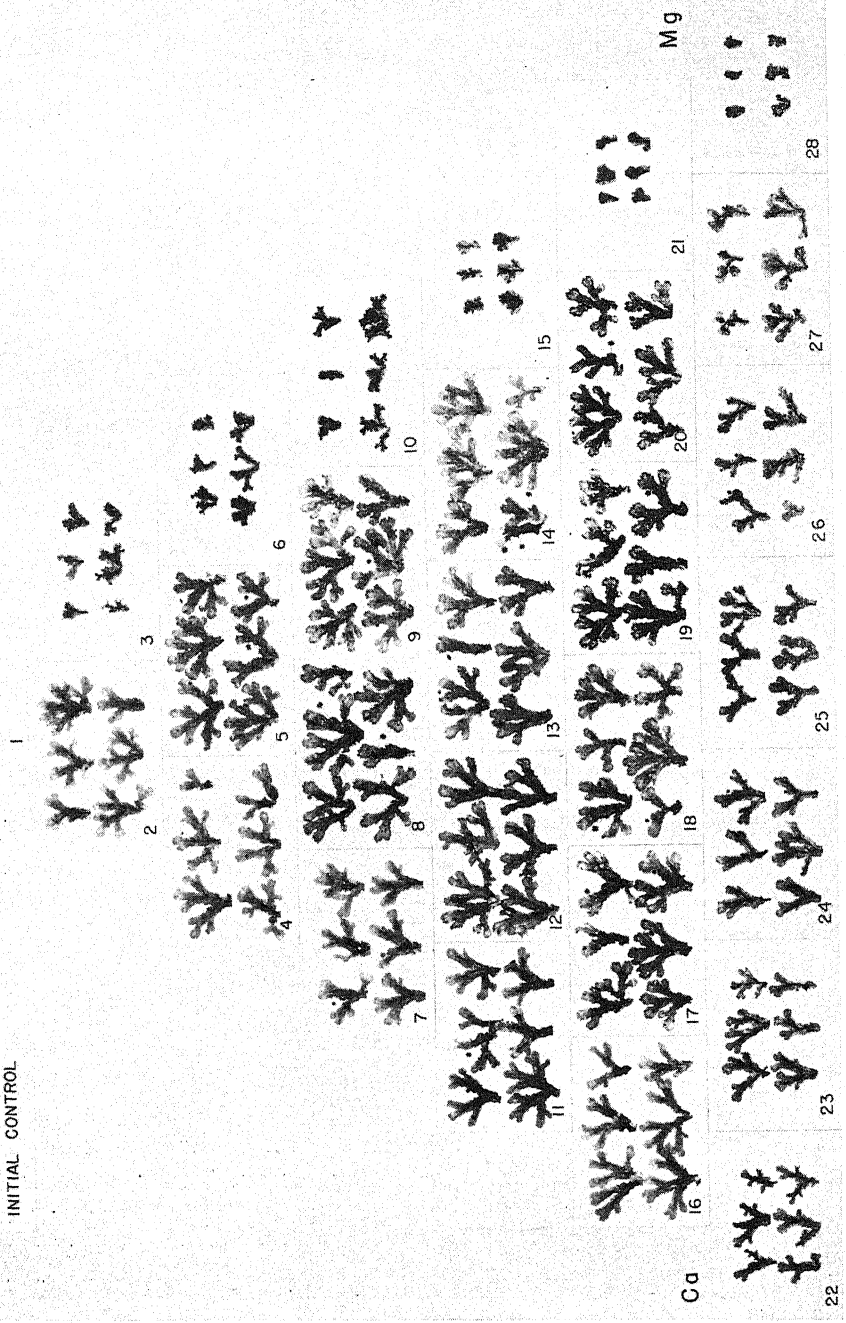


FIG. 3.—Cation triangle of experiment 8, April, 1940; antheridial plants on long photoperiod

EXP. 8

8 PLANTS

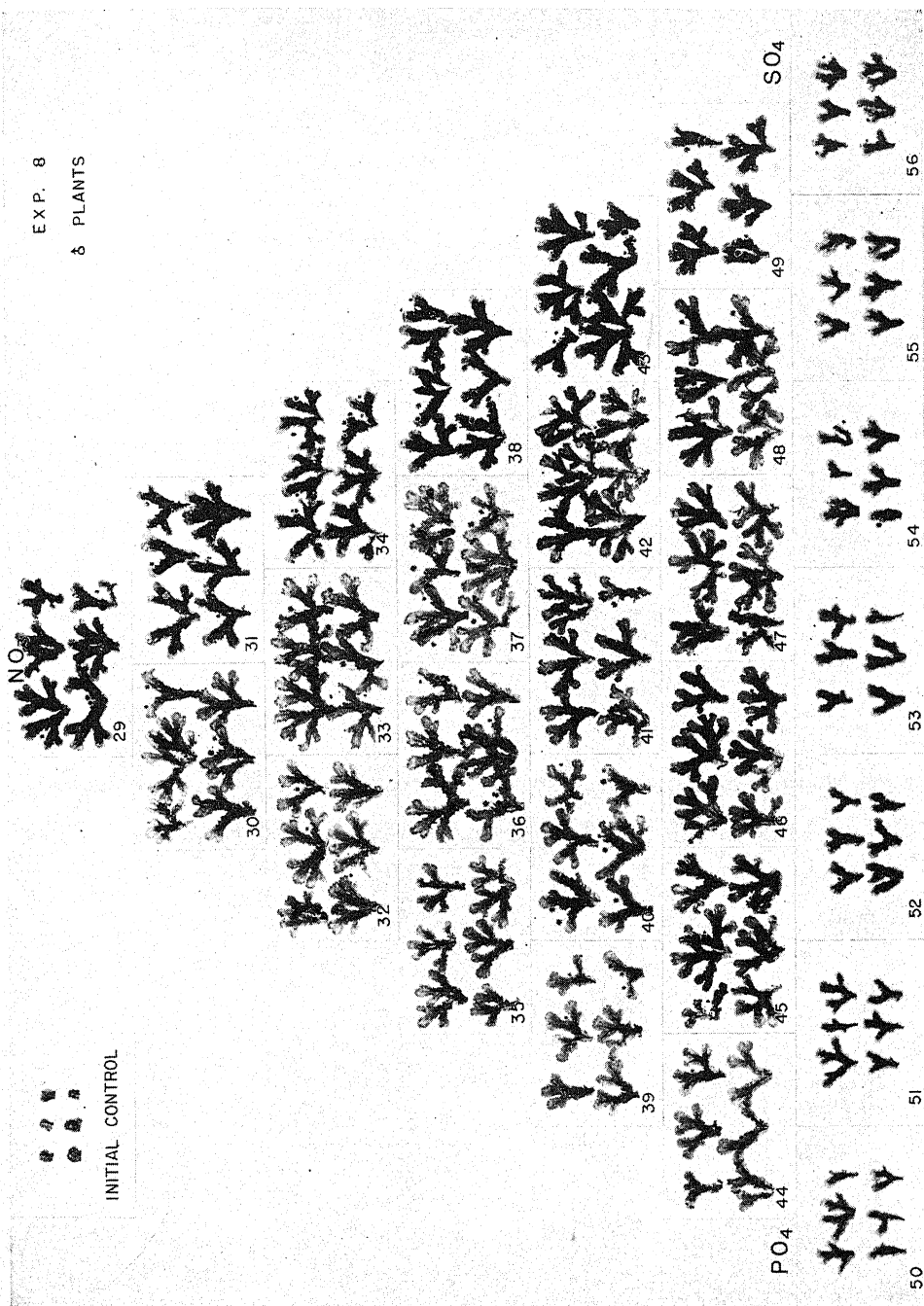


FIG. 4.—Anion triangle of experiment 8, April, 1940; antheridial plants on long photoperiod

ber of gemmae cups and of antheridiophores are given in tables 2 and 3. Certain of the plants possessed only a few gemmae cups, and these occupied a position in

TABLE 2

GROWTH OF MARCHANTIA POLYMORPHA ON NUTRIENT SOLUTIONS CONTAINING VARIOUS PROPORTIONS OF CATIONS OR ANIONS. ALL PLANTS GROWN ON LONG PHOTOPERIOD

POSITION IN TRI- ANGLE	WEIGHT OF 6 PLANTS (GM.)				RANK OF VARIOUS SOLUTIONS IN EACH TRIANGLE ON BASIS OF DRY WEIGHT OF 6 PLANTS		AREA OF 6 PLANTS (SQ. CM.)		NO. OF GEMMAE CUPS ON 6 PLANTS		NO. OF GAMETANGIOPHORES ON 6 PLANTS	
	EXP. 8		EXP. 9				EXP. 8	EXP. 9	EXP. 8	EXP. 9	EXP. 8 ANTHERIDIOPHORES	EXP. 9 ARCHEGONIOPHORES
	FRESH	DRY	FRESH	DRY	EXP. 8	EXP. 9						
	CATIONS K, CA, AND MG VARIED PROPORTIONATELY ON BASIS OF SIXTHS (CF. TABLE I) ANIONS PRESENT IN CONSTANT RELATIVE PROPORTION											
1.....	2.88	0.257	2.72	0.095	25	28	47	34	3	0	0	0
2.....	8.69	0.587	6.32	0.356	14	21	158	103	81	0	4	3
3.....	3.32	0.283	3.51	0.302	24	22	50	60	9	0	1	4
4.....	7.06	0.581	7.23	0.523	15	14	145	129	50	6	15	1
5.....	13.36	0.780	19.94	0.917	4	10	186	324	126	66	16	1
6.....	3.40	0.309	4.38	0.272	23	25	55	70	0	0	4	1
7.....	7.76	0.601	7.23	0.493	13	17	137	122	27	1	9	4
8.....	12.93	0.848	21.38	1.155	1	4	201	375	126	9	7	0
9.....	12.42	0.737	21.27	1.046	7	8	230	381	104	21	8	2
10.....	3.49	0.317	6.95	0.487	22	18	65	105	2	7	3	4
11.....	9.07	0.746	6.82	0.607	6	13	161	134	27	0	6	10
12.....	11.55	0.813	18.62	1.123	3	5	219	315	86	3	1	0
13.....	10.48	0.678	16.53	0.980	10	9	184	286	97	6	8	5
14.....	10.21	0.667	23.54	1.314	11	2	200	442	83	41	4	2
15.....	1.71	0.153	3.38	0.273	26	24	40	56	2	0	0	2
16.....	8.15	0.703	7.86	0.619	9	12	167	136	2	0	1	8
17.....	10.45	0.825	16.18	1.060	2	7	197	283	47	0	11	6
18.....	10.46	0.718	18.75	1.123	8	6	178	374	57	0	12	5
19.....	11.16	0.763	27.33	1.741	5	1	192	496	85	14	5	4
20.....	8.76	0.654	21.33	1.297	12	3	170	370	58	13	6	7
21.....	1.27	0.132	2.51	0.200	27	26	25	37	1	0	1	1
22.....	3.68	0.337	2.54	0.283	20	23	73	36	5	0	3	6
23.....	4.70	0.403	5.09	0.473	18	19	95	87	3	0	1	4
24.....	5.76	0.477	7.58	0.697	16	11	112	141	9	0	6	7
25.....	4.02	0.404	5.83	0.494	17	16	97	105	0	4	8	0
26.....	3.49	0.328	7.27	0.518	21	15	80	134	9	2	11	4
27.....	4.18	0.339	5.00	0.423	19	20	87	94	28	0	10	0
28.....	1.00	0.102	1.94	0.164	28	27	25	25	0	0	0	0
Totals.	.....	.....	.....	.....	.....	.....	3576	5254	1127	193	161	91

the older portion of the thallus and may have been differentiated at the time the cutting was made. Other plants possessed a large number, and most of these were present in the younger areas. A discussion of the results is deferred for comparison with the results of experiment 9.

EXPERIMENT 9.—On April 26, an experiment using cuttings from archegonial plants as described for experiment 8 was begun.

TABLE 2—Continued

POSITION IN TRI- ANGLE	WEIGHT OF 6 PLANTS (GM.)				RANK OF VA- RIOUS SOLU- TIONS IN EACH TRIANGLE ON BASIS OF DRY WEIGHT OF 6 PLANTS		AREA OF 6 PLANTS (SQ. CM.)		NO. OF GEM- MAE CUPS ON 6 PLANTS		NO. OF GAME- TANGIOPHORES ON 6 PLANTS	
	EXP. 8		EXP. 9				EXP. 8	EXP. 9	EXP. 8	EXP. 9	EXP. 8 AN- THERI- DIO- PHORES	EXP. 9 AR- CHEGO- NIO- PHORES
	FRESH	DRY	FRESH	DRY								
					EXP. 8	EXP. 9						
	ANIONS NO <sub>3</sub> , PO <sub>4</sub> , AND SO <sub>4</sub> VARIED PROPORTIONATELY ON BASIS OF SIXTHS (CF. TABLE 1) CATIONS PRESENT IN CONSTANT RELATIVE PROPORTION											
29.....	10.48	0.755	12.26	0.720	8	14	196	202	96	89	21	4
30.....	12.52	0.795	33.66	1.861	6	1	194	532	52	30	13	1
31.....	9.91	0.745	12.79	0.794	11	13	183	234	96	97	16	4
32.....	12.59	0.806	21.62	1.142	5	5	210	370	62	22	8	3
33.....	14.38	1.012	29.06	1.755	1	2	230	507	69	18	11	0
34.....	10.10	0.727	14.21	0.926	15	9	205	252	105	50	31	16
35.....	8.86	0.577	8.83	0.529	19	20	170	167	11	1	2	4
36.....	12.80	0.837	15.63	0.880	4	10	236	284	62	0	11	5
37.....	11.15	0.919	21.91	1.258	2	4	234	401	64	10	19	2
38.....	9.56	0.738	16.17	1.069	13	7	197	280	86	6	19	6
39.....	10.94	0.686	14.50	0.874	16	11	142	250	20	1	10	5
40.....	8.78	0.753	13.29	0.811	9	12	175	264	47	0	14	5
41.....	8.55	0.610	17.61	1.090	18	6	173	325	29	0	8	8
42.....	11.72	0.745	22.36	1.353	10	3	244	405	75	0	5	10
43.....	11.13	0.851	8.72	0.523	3	21	220	172	84	48	30	0
44.....	7.48	0.513	11.30	0.678	21	16	150	174	22	0	16	10
45.....	9.50	0.740	8.08	0.595	12	19	208	165	25	0	7	8
46.....	9.28	0.629	9.96	0.689	17	15	204	188	14	0	5	11
47.....	10.31	0.734	9.33	0.596	14	18	211	189	34	1	6	19
48.....	10.32	0.783	14.57	1.039	7	8	215	288	64	0	11	10
49.....	7.21	0.559	9.07	0.601	20	17	158	174	44	2	18	11
50.....	3.23	0.265	3.05	0.220	25	24	80	47	4	0	9	3
51.....	3.41	0.230	3.35	0.219	26	25	88	50	2	0	7	14
52.....	2.93	0.206	2.57	0.181	28	27	75	42	2	0	5	6
53.....	3.09	0.222	2.38	0.172	27	28	80	44	2	0	4	2
54.....	3.46	0.286	2.57	0.204	24	26	77	48	1	0	9	7
55.....	3.98	0.343	2.95	0.234	22	23	90	71	1	0	15	4
56.....	3.63	0.331	3.18	0.250	23	22	80	67	0	0	15	5
Totals.....							4725	6192	1173	375	345	183

On May 19, the plants under conditions of long photoperiod and supplied with solution 1 were watery in appearance, and some of them became infected with molds. Most of these plants were practically dead at this time. These symptoms may be attributable to the presence of a high concentration of potassium or to the combined absence of magnesium and calcium. On this same date, the plants



supplied with solution 22 were much smaller than most of the other plants and were dark green in color, streaked with black or deep red along the midribs. The older portion of the thallus was streaked with yellow-green. The plants supplied with solution 28 had grown very little up to this date, were black in color, and all were dead. Plants supplied with solution 21 had some of the symptoms apparent in those plants supplied with solution 28, although they had not progressed so far. The plants supplied with solution 15 showed some symptoms similar to those developed in 21 and 28. Apparently, if calcium is lacking, a high concentration of magnesium—together with a low concentration of potassium—is definitely toxic. Those plants supplied with solutions lacking in potassium (that is, supplied with solutions 22 to 28, inclusive) developed characteristic symptoms. The margins of the older portion of the thallus turned light tan to brown, finally becoming watery in appearance, and ultimately the margins became dry. Progressive stages of this symptom were characterized by concentric rings, each ring marked by a slightly darker brown color, particularly noticeable in the notches between the branches of the thallus. Those plants supplied with solutions lacking in calcium (solutions 1, 3, 6, 10, 15, 21, and 28) developed more or less characteristic symptoms, as described for experiment 8. In this particular group, plants on solution 10 grew the most. There was some evidence of regeneration of the thallus.

Those plants supplied with solutions lacking in magnesium (solutions 1, 2, 4, 7, 11, 16, and 22) did not develop any characteristic symptom. Plants supplied with solution 22 produced very little growth, and there was some burning in the margins of the plants supplied with solutions 2, 4, 7, 11, and 16. The thallus of plants supplied with solution 1 had extensive burning at the margins and grew very slightly. On this date all the plants in the central portion of the triangle were about equal in gross appearance.

On May 19, the plants supplied with solutions of the anion triangle were also carefully examined. Those supplied with solutions lacking in nitrates (solutions 50 to 56, inclusive) were all small and had produced only enough growth approximately to double the original area of the cuttings. All these plants were pale yellow-green in color, and the scales on the ventral surface were bright red. This color could be seen showing through the more or less transparent thallus. The tips of the thalli were light green, and branches were somewhat narrow at the tip. Most extensive growth was produced by those plants supplied with solution 56, and decreased growth was apparent in order of solution number, down to solution 50. It was noted, in caring for these plants lacking in nitrates, that rhizoids developed in abundance very early after starting the experiment. Those plants supplied with solutions lacking in phosphorus (solutions 29, 31, 34, 38, 43, 49, and 56) were dark green and produced an abundance of dark-colored rhizoids. The midribs were al-

most black, with a slight reddish hue. Except for those plants supplied with solution 56, which were much smaller than the others, all the plants in this group were practically alike in size and produced about as much growth as did the plants in other positions in the triangle, with the exception of those mentioned immediately below. Those plants supplied with solutions 30, 33, 37, 42, and 48 appeared at this time to have made much more growth than the other plants in the triangle. The only characteristic of this particular group which would account for their similarity in gross appearance at this time is the fact that all these solutions contained one-sixth of the  $\text{PO}_4$  solution. This might indicate that maximum growth of *Marchantia* is associated with low concentration of phosphorus, which is at variance with the results attained with thalli grown from gemmae (33). A plant growing on solution 30 increased in area from 2.5 to 160 sq. cm. in 32 days. All plants in this experiment were harvested on May 27 (figs. 5, 6, 7, 8).

#### DISCUSSION OF EXPERIMENTS 8 AND 9

The results of fresh and dry weight determinations of experiments 8 and 9 are recorded in tables 2 and 3. It is apparent that there were great differences in the amount of dry weight produced by various groups of plants supplied with the different solutions. On the basis of these dry weights, the rank of the various solutions was determined. Thus the solution with rank no. 1 produced the greatest amount of dry weight, and the solution with rank no. 28 produced the least, the other solutions ranking intermediately. The results of the counts on gemmae cup formation, antheridiophore and archegoniophore formation, and the area in square centimeters are recorded in tables 2 and 3.

The rate of accumulation of dry weight by *Marchantia* is markedly associated with the variations in nutrient solutions. In the cation triangle of both experiments 8 and 9, greatest growth was produced on the solutions in the central portion of the triangles. There was little change in the comparative rank of any given solution, regardless of the system used for calculation—whether on the basis of fresh weight or dry weight and of five or six plants. The responses of the antheridial plants are slightly different from those of the archegonial plants, but the correspondence is close considering the fact that the two experiments were carried out at different seasons of the year and under different conditions of intensity of daylight and average temperature.

On those solutions lacking in calcium the least growth was produced, and of this group the most growth was on solution 10, which contains about equal amounts of magnesium and potassium. As a group, it may be noted also that on those solutions lacking in potassium relatively little growth took place. A lack of magnesium did not affect the growth as much as the other absences, particularly in those solutions in which the calcium content was relatively high and the potassium content relatively low; for example, solutions 11 and 16.

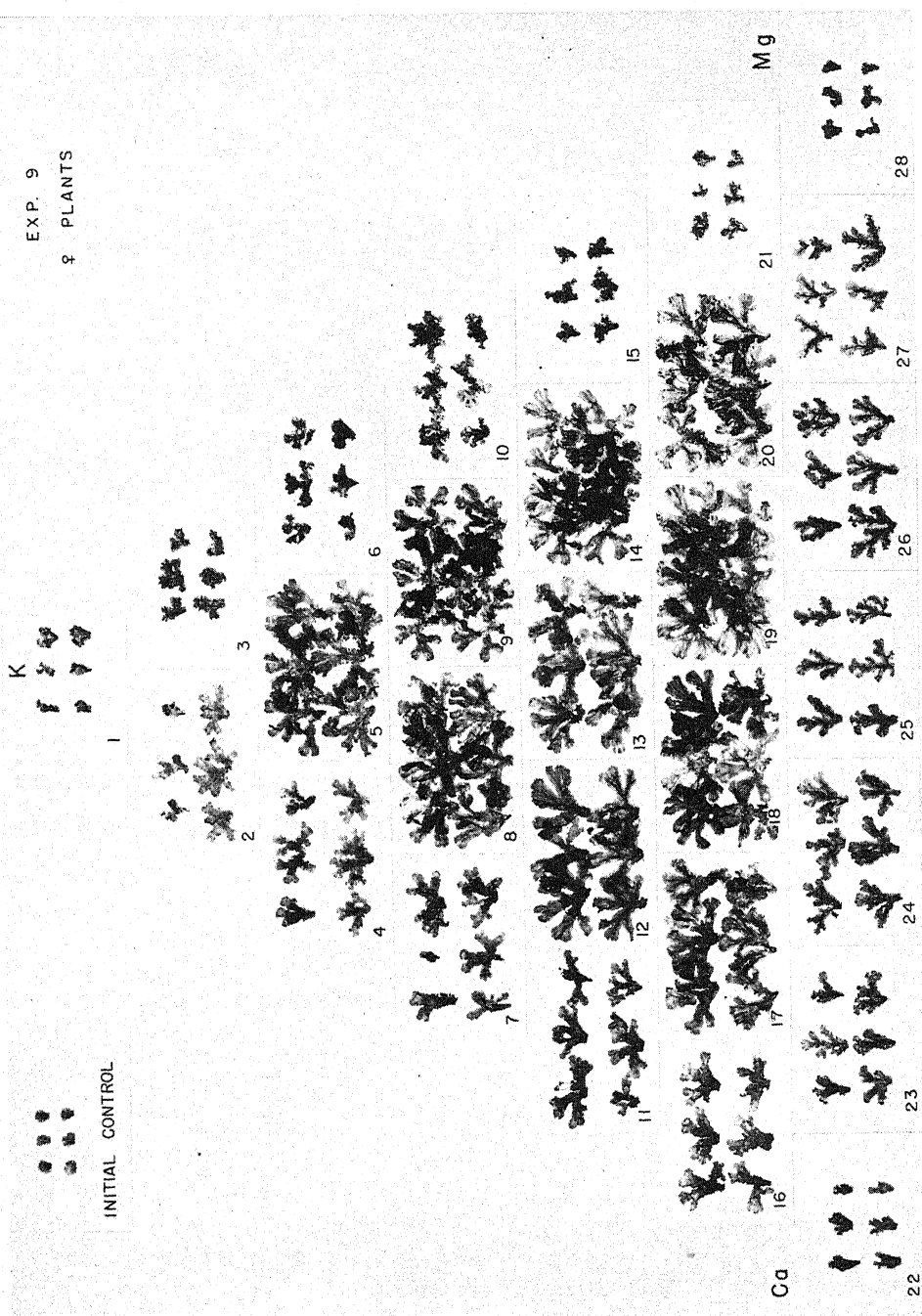


FIG. 5.—Cation triangle of experiment 9, May, 1940; archegonial plants on long photoperiod. Large amount of growth on some combinations necessitated overlapping the plants for photographing.



INITIAL CONTROL



FIG. 6.—Anion triangle of experiment 9, May, 1949; archegonial plants on long photoperiod. Large amount of growth on some combinations necessitated overlapping the plants for photographing.

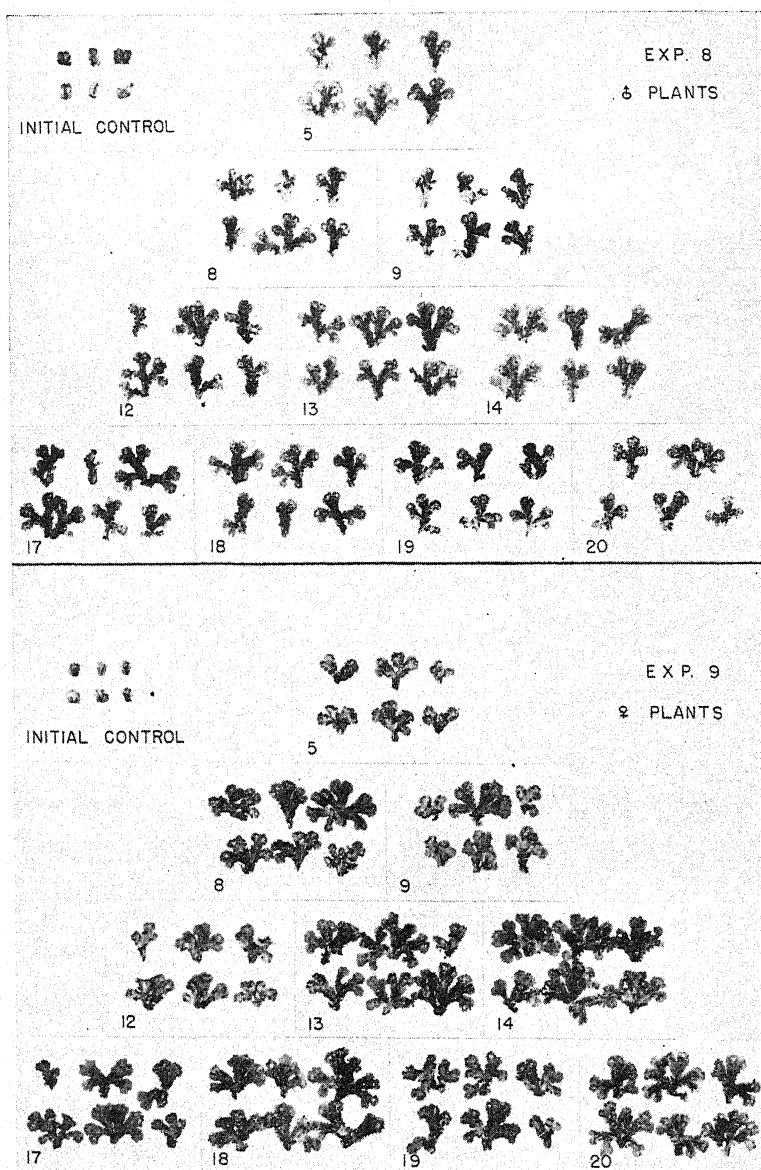


FIG. 7.—Cation triangles of experiments 8 and 9 on short photoperiod. Antheridial (above) and archegonial (below) plants grown April and May, 1940, respectively. Numbers correspond to same combinations as indicated in figs. 3 and 5. Total growth is much less than under long photoperiod (*cf.* figs. 3 and 5; tables 2 and 3).

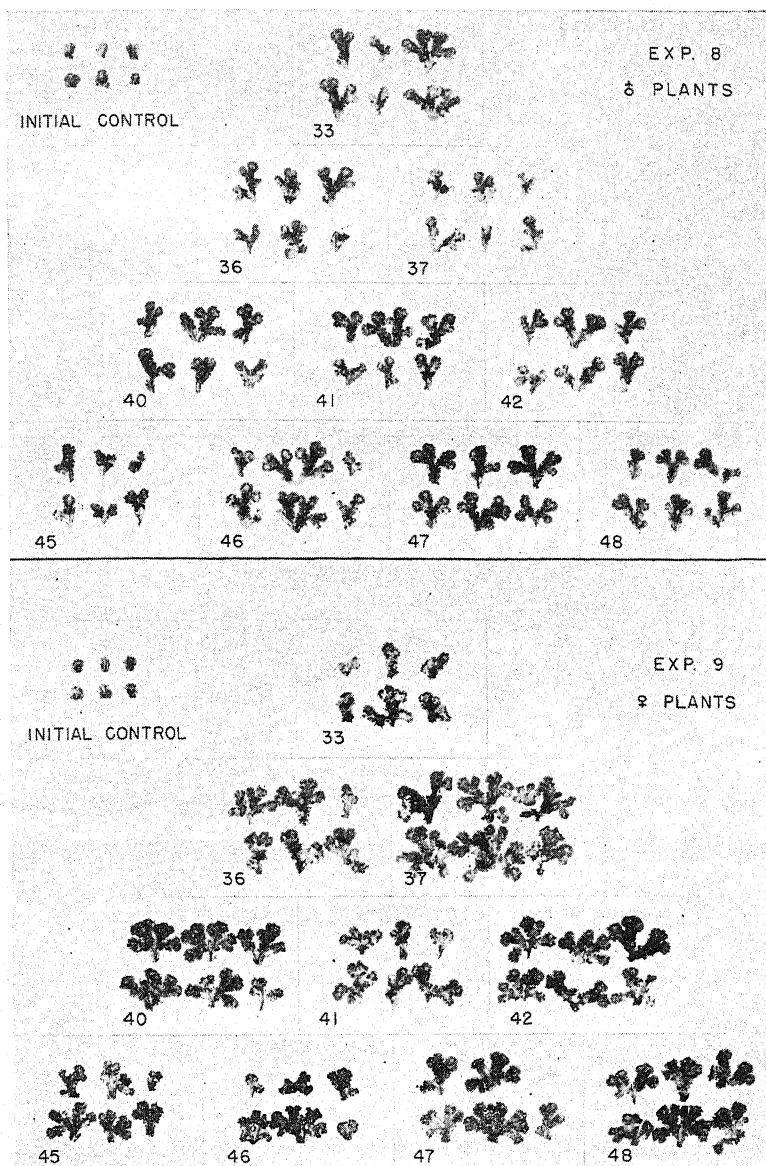


FIG. 8.—Anion triangles of experiments 8 and 9 on short photoperiod. Antheridial (above) and archegonial (below) plants grown April and May, 1940, respectively. Under short photoperiod the total growth is less than under long photoperiod.

In the anion triangles of both experiments there are several points of significance. In the first place, it is obvious that lack of nitrogen is accompanied by

TABLE 3

GROWTH OF MARCHANTIA POLYMORPHA ON NUTRIENT SOLUTIONS CONTAINING VARIOUS PROPORTIONS OF CATIONS OR ANIONS. ALL PLANTS GROWN ON SHORT PHOTOPERIOD

POSITION IN TRIANGLE	WEIGHT OF 6 PLANTS (GM.)				AREA OF 6 PLANTS (SQ. CM.)		NO. OF GEMMAE CUPS ON 6 PLANTS		NO. OF GAMETANGIOPHORES ON 6 PLANTS	
	EXP. 8		EXP. 9		EXP. 8	EXP. 9	EXP. 8	EXP. 9	EXP. 8 ANTHERIDIOPHORES	EXP. 9 ARCHEGONIOPHORES
	FRESH	DRY	FRESH	DRY						
	CATIONS K, CA, AND MG VARIED PROPORTIONATELY ON BASIS OF SIXTHS (CF. TABLE 1) ANIONS PRESENT IN CONSTANT RELATIVE PROPORTION									
5.....	5.91	0.330	4.87	0.250	91	88	110	64	0	6
8.....	4.67	0.271	9.55	0.509	94	165	86	153	0	1
9.....	3.94	0.239	7.20	0.376	93	116	83	75	0	1
12.....	5.04	0.365	6.50	0.354	113	112	98	52	2	3
13.....	7.01	0.451	11.98	0.670	137	194	143	177	0	0
14.....	6.56	0.445	13.52	0.854	133	238	120	223	0	2
17.....	6.80	0.460	6.94	0.441	125	137	116	110	0	0
18.....	5.65	0.378	11.48	0.732	112	202	108	172	0	4
19.....	4.66	0.294	7.60	0.451	101	149	81	98	0	0
20.....	4.03*	0.274*	10.41	0.704	87*	201	90*	188	0*	2
Totals.....					1086	1602	1035	1312	2	19
	ANIONS NO <sub>3</sub> , PO <sub>4</sub> , AND SO <sub>4</sub> VARIED PROPORTIONATELY ON BASIS OF SIXTHS (CF. TABLE 1) CATIONS PRESENT IN CONSTANT RELATIVE PROPORTION									
33.....	3.64	0.257	3.76	0.226	90	70	53	31	0	3
36.....	2.54	0.191	5.30	0.344	68	114	44	58	2	2
37.....	1.60	0.127	11.12	0.637	50	208	38	183	0	2
40.....	3.23	0.185	7.70	0.427	77	155	58	76	1	3
41.....	3.15	0.215	4.09	0.227	128	93	44	31	0	3
42.....	3.61	0.199	7.69	0.437	78	155	49	109	0	5
45.....	1.88	0.118	3.62	0.250	45	79	12	42	3	2
46.....	4.32	0.282	3.28	0.199	125	76	53	19	1	0
47.....	5.15	0.296	5.55*	0.372*	107	113*	75	76*	0	3*
48.....	3.08	0.211	6.94	0.418	75	138	57	109	4	3
Totals.....					843	1201	483	734	11	26

\* Triangle position 20 in experiment 8 and triangle position 47 in experiment 9 possessed only 5 plants each at time of harvest. Had 6 plants been present it is assumed that all figures given would have been about one-fifth greater.

only slight growth, regardless of the content of phosphate or sulphate. It is also obvious that most growth occurred on those solutions low in phosphate and high in nitrate; that is, in solutions 30, 33, and 37. Considerable growth was also pro-

duced on solutions 42 and 48. Thus apparently a more marked effect was evident in relation to extremely small variations in the phosphate content than to any other single anion. The rate of accumulation of dry matter was apparently favored by low phosphorus content. Abundant phosphorus, particularly in those solutions where the nitrate content was low, was associated with decreased growth. Lack of sulphur resulted in decrease in the rate of growth on those solutions where the phosphate content was high but did not greatly affect the rate of growth on those solutions where the phosphate content was low (and the nitrate content high); for example, solutions 29 and 30.

More gemmae cups were produced on the antheridial than on the archegonial plants in both triangles (table 2). In the cation triangles more gemmae cups were produced on the solutions occupying a central position, particularly solutions 5 and 9, which were relatively low in calcium and magnesium and relatively high in potassium. Fewer gemmae cups were produced when there was an absence of potassium. In the anion triangles the production of gemmae cups was strikingly correlated with the phosphorus content of the solution. In both experiments more gemmae cups were produced on the solutions lacking in phosphorus than on any other, and the fewest gemmae cups were produced on the solutions lacking in nitrogen. There seemed little correlation between gemmae cup formation and sulphur content of the solution. Although in many combinations in both experiments numerous gemmae came to rest on the glass cloth and were permitted to remain relatively undisturbed for many days, none grew to form a mature thallus. Most gemmae disintegrated or were washed off the cloth when the solutions were changed.

The number of antheridiophores or archegoniophores produced on the various solutions was not clearly correlated with the concentration of any particular ion. In both experiments these structures were produced throughout both triangles. In the anion triangle of experiment 8 there seemed to be more antheridiophores on the solutions lacking phosphorus than on any other, although the correlation is not high. The determining factor in formation of antheridiophores and archegoniophores seems to be length of photoperiod rather than character of nutrient solution.

The total area produced by the plants growing on the various solutions was correlated rather closely with the accumulation of dry weight (tables 2, 3). Also, much greater growth was produced on certain solutions in the anion triangles of both experiments than was produced anywhere in the cation triangles. This is particularly true for solutions 30, 33, and 37, such solutions being relatively low in phosphorus. It may be that the phosphorus content of the cation triangles was slightly too high for maximum growth. If one wished to find a nutrient solution most favorable for the growth of this particular plant, consideration of the various



results recorded here would indicate that such a solution should have a relatively low phosphorus content and a relatively high nitrogen content. But to produce the maximum development of antheridiophores or archegoniophores, or of gemmae cups, it might be necessary to utilize in the nutrient solution an amount of phosphorus approximating 0.00015 mol per liter or less.

#### COMPARATIVE EFFECTS OF LONG AND SHORT PHOTOPERIOD ON GROWTH OF MARCHANTIA

As already noted, at the time experiments 8 and 9 were started, comparable plants were placed on solutions 5, 8, 9, 12, 13, 14, 17, 18, 19, and 20 and solutions 33, 36, 37, 40, 41, 42, 45, 46, 47, and 48, and exposed to conditions of short photoperiod. The area, fresh and dry weight accumulation, gemmae cup formation, and gametangiophore production are recorded in table 3.

The growth and accumulation of dry matter by these plants was much less than under conditions of long photoperiod. Practically no antheridiophores or archegoniophores were produced by any of the plants exposed to short photoperiod, and those which were produced were usually found in the older portions of the thalli and were probably already initiated at the time the experiment was started. The area of these plants paralleled closely their relative rate of accumulation of dry weight and was much less than comparable plants under conditions of long photoperiod. All plants exposed to short photoperiod produced an abundance of gemmae cups, and while the total number of cups produced by any particular set was in certain cases about the same as those produced by comparable plants exposed to long photoperiod, the number produced per unit area was always far greater. On most solutions the total number of gemmae cups produced by any particular group of six plants—growing on a specific nutrient solution and exposed to short photoperiod—far exceeded comparable plants exposed to long photoperiod. This is true in spite of the fact that the total area of these plants and their total dry weight were much less. On short photoperiod archegonial plants produced a greater total number of gemmae cups than did antheridial plants (table 3).

#### INTERNAL ANATOMY

In experiments 8 and 9, one of the six thalli of each treatment was transferred to killing fluid immediately after the fresh weight of the lot was determined. After fixation, a representative thallus fork was selected for anatomical study. A piece of plant body, extending about 4 mm. from the apical notch toward the base of the plant, was trimmed so that its width approximated 2.5 mm. Immediately adjacent, a cross section of the thallus approximately 5 mm. wide was removed. Both plant fragments were imbedded in paraffin and sectioned at 7  $\mu$ . All sections were vertical; those through the apex longitudinal, and those through the more nearly mature part of the plant were transverse with respect to the axis of the thallus. The

longitudinal sections had in most instances meristematic, maturing and mature tissues. The cross sections had only relatively mature cells and tissues. Close microscopic examination of all preparations was made to determine whether any differences were apparent and whether such differences were possibly related to variations in nutrient supply and photoperiod.

Aside from such differences as width of thallus, coloration of scales and lower epidermis, development of rhizoids, and rapidity of dichotomy—characteristics which may be more readily determined by gross examination—very few striking anatomical differences were observable in the microscopic sections. Whenever the apical region of *Marchantia* was active mitotically, the number of cell divisions of the respective segments of the apical cells was about constant. Cell counts were made on both types of sections on nearly all the preserved plants. Although the thickness of the thallus varied considerably, the number of cells in a cross section was relatively constant. Counts were made vertically in a region twenty cells back of the apical cells in the longitudinal sections and in the midrib of the cross sections. Few thalli deviated from a cell count of 16 to 20; the greatest variation was from 11 to 26. Only the storage tissue was included in the count, to obviate the difficulties in recognizing cells in the photosynthetic areas in necrotic thalli. Plants growing on short photoperiod or on a solution deficient in one or two ions were thin vertically compared with plants growing on long photoperiod and a solution containing all the ions used in this experiment. Such thinner plants almost invariably possessed as many cells vertically as those thalli which appeared to be thick, the difference being in the size of the cells. Plants growing on solutions where necrosis occurred were thin in vertical distance and in the number of cells as counted vertically.

#### ANATOMICAL EFFECTS OF NUTRIENT DEFICIENCIES

The results of anatomical studies are based on more than 1000 slides of serial sections. On long photoperiod some plants received solutions lacking in one or more cations or anions.

**POTASSIUM DEFICIENCY.**—Plants grown in solutions very low in potassium possess no single anatomical alteration or condition attributable to such deficiency. Plants from solutions of the cation triangle numbering 22 to 28 inclusive (fig. 2) showed the customary anatomical development when a balance between calcium and magnesium was maintained. Necrosis was very marked in 22 (fig. 10A) and 28 (fig. 12C), the former lacking in calcium and the latter in magnesium (in addition to the absence of potassium), where large areas or nearly the entire thallus was nonliving (fig. 12C). Local necrosis was evident in 23 and 24 in female plants (grown in May). Plants on solutions 25, 26, and 27 were not necrotic except for the lower tip of the female plant in longitudinal section on solution 26. Anatomical

cally the contrast between the plants on combination 27 and the nearly lifeless plants in position 28 is most striking. The former received one-sixth of the calcium solution and five-sixths of the magnesium solution, while the latter received the six-sixths magnesium solution only. In general, plants in the row lacking potassium stored more starch than plants in any other treatment, with the possible exception of the plants in the row lacking magnesium. Cell walls were not noticeably thick, but no plasmolysis occurred during fixation. Cells of the photosynthetic filaments were relatively large, distended, and possessed few chloroplasts. Pegged and smooth rhizoids were abundant.

**CALCIUM DEFICIENCY.**—The most striking anatomical changes were observed in relation to calcium deficiency. Apical growth in such plants ceases after a few days, and the usually green growing point becomes translucent and soon blackens. Large areas of such plants (1, 3, 6, 10, 15, 21, and 28) are necrotic, possess few rhizoids, and regenerate new thalli from the lower surface in the region of the midrib. Since regeneration was induced only in the plants lacking calcium, and since the development of new thalli occurs with such regularity, details will be discussed later in this paper. Death of the tips of the thallus was most rapid in combinations 21 and 28, which received relatively large percentages of magnesium. Regeneration occurred with greater regularity in 1, 3, and 6, becoming less frequent in 10, 15, and 21. Only one plant on combination 28 regenerated a thallus. Plants lacking in calcium generally possessed large upper epidermal and relatively few photosynthetic cells, which remained distended even when a layer of algae (mainly *Chlamydomonas*) completely covered the upper surface of the plant. These cells of the photosynthetic layer usually were devoid of chloroplasts.

**MAGNESIUM DEFICIENCY.**—Plants grown on combinations 1, 2, 4, 7, 11, 16, and 22 showed magnesium deficiency. The first and last combinations were also lacking in calcium and potassium, respectively. Plants in both treatments were necrotic and on combination 1 regenerated. Plants on the remaining solutions comprising the row in which magnesium was lacking were comparable with control plants in appearance, except that the mature upper epidermis and the mature photosynthetic layers were collapsed. Cells of these regions were well fixed when near the growing point, and immature. As seen in longitudinal sections there was a gradient of turgid cells to collapsed ones in the upper part of the thallus. Starch storage was pronounced in all treatments. The lower epidermis was thick walled and stained readily with safranin.

**NITROGEN DEFICIENCY.**—Characteristic growth responses were evident in the lowest row of the anion triangle. All plants were stunted, were light yellow in hue and deep red or purplish red underneath. This opaque red color was present in all the older scales and in the lower layers of the thallus, except the last 5 mm. of the tip, which was fairly translucent. The red color persisted after plants were pre-

served for nearly a year in F.A.A. solution (fig. 9A). The aquatic form or variety of *Marchantia* is described as possessing on the upper surface a median band pigmented with purple, associated with the absence of air chambers (7). Colored cell walls in liverworts have been ascribed to strong light combined with rather high temperature (11). MÖBIUS (25) reports that anthocyanin is present in the cell walls of *Marchantia* and persists for many years, even in a glycerin mount.

In longitudinal section (fig. 10B) the rather short zone of meristematic cells at the tip passes rather abruptly into the mature region of the thallus, which is characterized by large storage cells. Plants on a solution lacking in nitrogen were the first to develop rhizoids and were firmly attached to the glass cloth in a few days. No microscopic evidence was found of initiation of rhizoids nearer the tips. Dichotomy in plants lacking in nitrogen was infrequent, so that the narrowly elongated thalli were easily recognizable. Cross sections usually possess only one midrib (fig. 9A). Stored starch was evident in only a few of the plants, and all cell walls were thicker than in plants receiving all ions. The plant selected from combination 50 in experiment 9 was the only one showing a necrotic area among the anion plants. In a few scattered combinations, such as 10 and 16 in experiment 8 and 48 in experiment 9, under long photoperiod the lower epidermis and a few adjacent layers were relatively thick walled, but not as prominently so as in the nitrogen deficient plants.

PHOSPHORUS DEFICIENCY.—In these experiments plants grew well and to large size on solutions containing very small amounts of phosphorus. In fact there was considerable development, even though the solution contained no phosphorus—apparently a sufficient amount being present in the growing tips used for purposes of propagation at the beginning of the various experiments. The midribs of the thalli appeared black. Near harvest time it was obvious that the black coloration was a combination of the red lower surface and the dark green of the rest of the thallus. The older scales and the lower part of the thallus were red, much as in the nitrogen deficient plants, but possibly more intense. This color also persisted in F.A.A. solutions and in freehand sections was found to be limited to scales, rhizoids, lower epidermis, and at most the three cell layers nearest the lower epidermis. In the absence of fresh material it is presumed that the red color is the same for the plants when nitrogen or phosphorus is absent. The number of smooth rhizoids was very abundant (fig. 10C). In the midrib region pegged rhizoids often formed a bundle having a diameter which exceeded the width of the thallus. In the storage region of some plants lacking phosphorus (in experiment 9) cells were apparently plasmolyzed (fig. 10C).

SULPHUR DEFICIENCY.—Combinations 29, 30, 32, 35, 39, 44, and 50 contained no sulphur. Solutions 29 and 50 also lacked phosphorus and nitrogen, respectively, and the plants possessed the anatomical characteristics of these deficiencies.

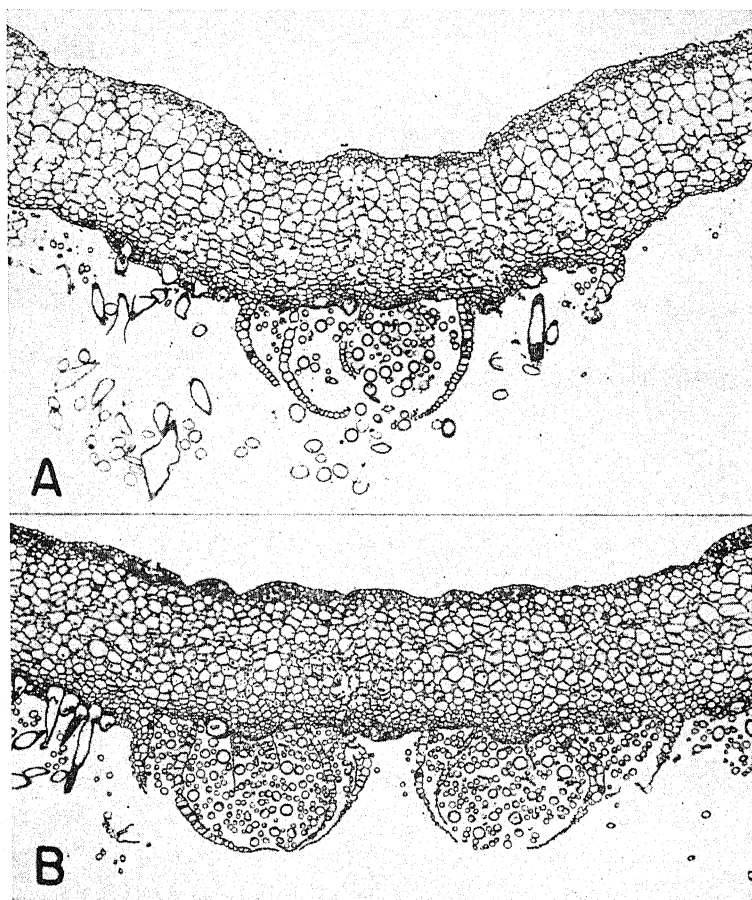


FIG. 9.—Cross sections of thalli in midrib region: *A*, nitrogen deficient plant on long photoperiod, experiment 8, on solution 55; cells of scales, rhizoids, lower epidermis, and adjacent layers are thick walled and colored red. *B*, initial control plant; darker staining cells below photosynthetic layer of thallus shown enlarged in fig. 144.

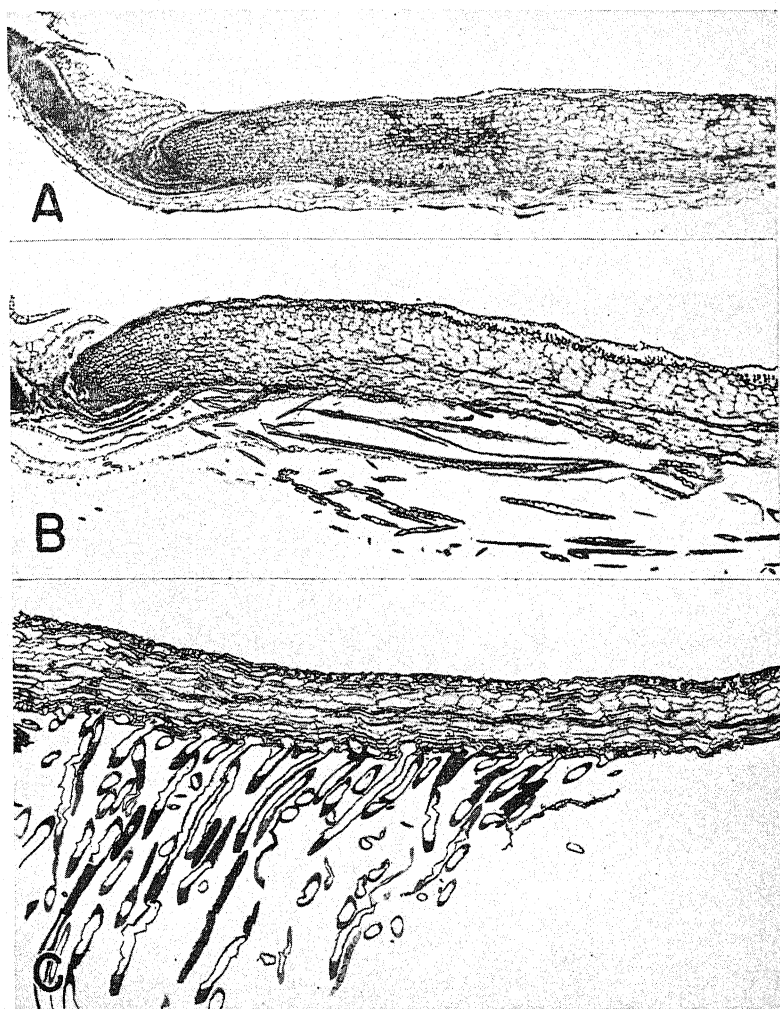


FIG. 10.—Vertical, longitudinal sections of thalli in midrib region: *A*, plant grown on combination 22 lacking potassium and magnesium but having relatively high concentration of calcium; experiment 8 on long photoperiod; several necrotic areas visible. *B*, plant grown on combination 54 lacking nitrates; experiment 9 on long photoperiod; mature region passes abruptly into less differentiated zone near tip. *C*, plant grown on combination 34 lacking phosphates; experiment 9 on long photoperiod; smooth rhizoids numerous.

Cross and longitudinal sections of plants on solutions 30, 32, 35, 39, and 44 resemble the sections of initial control plants and plants located in combinations in the center of the anion triangle in all respects. Apparently no sulphur deficiency existed, or such deficiency is not shown in anatomical details.

#### EFFECTS OF EXCESS IONS

Because of the nature of the experimental conditions, the greatest concentration of any ion was at the apices of the triangles (fig. 2). Combinations 1, 22, 28, 29, 50, and 56 contained the highest concentrations of the respective cations or anions, but each also was lacking in two ions. Limitation and alteration of growth and anatomical changes may not be ascribed to excess ions, unless adjacent combinations with a full complement of ions in some proportion also have similar characteristics. If comparable conditions are not present in the latter, it is reasonable to assign characteristics of growth and structure in the plants at the apices of the triangle to lack and not to excess of ions.

In the cation triangle, combinations with all ions present and with potassium, calcium, and magnesium in highest concentration were 5, 17, and 20, respectively. Plants grown on these combinations approximated the control plants in gross appearance and internal anatomy. Similarly, combinations 33, 45, and 48 contained all ions, and nitrates, phosphates, and sulphates were present in the largest amounts, respectively. Plants grown on these solutions also resembled the control or the original stock plants. Nutrient solutions of greater salt content, and consequently greater osmotic concentration, could be employed. Any appreciable anatomical change in plants grown on the combinations just discussed could be attributed to excesses if similar conditions were not found in plants receiving a smaller quantity of a particular ion. No anatomical effects of excess ions were observed in microscopic preparations of plants of experiments 8 and 9.

#### INTERACTION OF IONS

In combination 22, which was high in relative concentration of calcium ions, necrotic areas were present throughout the thallus (fig. 10A). Similar but less marked necrosis was also present in plants grown on solutions 23 and 24. These also were on relatively high calcium solutions, five-sixths and four-sixths, respectively. Plants on combination 26 were nearly devoid of necrosis, and thalli on 25 and 27 lacked necrotic areas entirely. Plants on solution 28 were almost wholly necrotic (fig. 12C). Since all combinations (22 to 28) lacked potassium, such diversity of anatomical expression cannot be ascribed to its absence alone. These combinations varied conversely in calcium and magnesium content, and it is likely that high calcium supply accompanied by low magnesium supply—together with lack of potassium—favored necrosis. High magnesium supply accompanied with

varied calcium supply (one-sixth to three-sixths) and absence of potassium favored the usual anatomical development in *Marchantia*.

In the row of nutrient combinations lacking calcium (1, 3, 6, 10, 15, 21, and 28), low magnesium supply and attendant high potassium supply favored thallus regeneration, but a nutrient supply with converse proportions of these cations retarded the formation of adventitious thalli.

No interrelationships of anatomical effect on plants were noted with the cation magnesium and the other cations, and none of the anions interacted to produce an anatomical change great enough to be observed.

#### ANATOMY OF PLANTS SUPPLIED WITH ALL NUTRIENT IONS IN VARIOUS PROPORTIONS

Plants on combinations 5, 8, 9, 12, 13, 14, 17, 18, 19, and 20 in experiments 8 and 9 are very similar anatomically. Such plants grew large in area, elongated rapidly (at times even elongating their gemmae cups), and possessed rather brittle thalli. As a rule the upper epidermis was shrunken in microscopic preparations, and the photosynthetic filaments were partially collapsed. Walls of cells in the central portion of the storage region were so thin that many cells collapsed, forming a strandlike zone running longitudinally through the thallus. Scales are formed at the apex of the plant but are shrunken and inconspicuous in the mature region. Both types of rhizoids develop as in control plants.

In the anion triangle, combinations receiving all ions in various proportions were 33, 36, 37, 40, 41, 42, 45, 46, 47, and 48. Again the anatomy of plants in experiments 8 and 9 is very similar. As a rule the cells and tissues in these plants were comparable with plants supplied with all ions in the cation triangle. No single characteristic nor any obvious set of criteria was found on which to separate plants in these twenty positions from one another. Antheridial plants in experiment 8 and archegonial plants in experiment 9 reacted similarly.

#### ANATOMICAL EFFECTS OF SHORT PHOTOPERIOD

Anatomically the effect of short photoperiod on plants in both triangles, in both experiments, was identical. Only the twenty combinations just mentioned were used on short photoperiod. All plants appeared thinner in vertical section than plants in similar positions on long photoperiod. Cell counts in cross section in the storage region averaged 16 to 20, a range that duplicates the cell count in plants on long photoperiod. In the storage region of these plants the upper 5-7 cell layers averaged  $40-50 \times 110-125 \mu$  per cell. Cells in the next 4-6 layers averaged  $20 \times 75-115 \mu$ . Cells nearest the lower epidermis and several layers above averaged  $10-20 \times 40 \mu$ . The long axis of all cells was parallel to the axis of the thallus. The uppermost layer of storage cells averaged larger in size than comparable cells



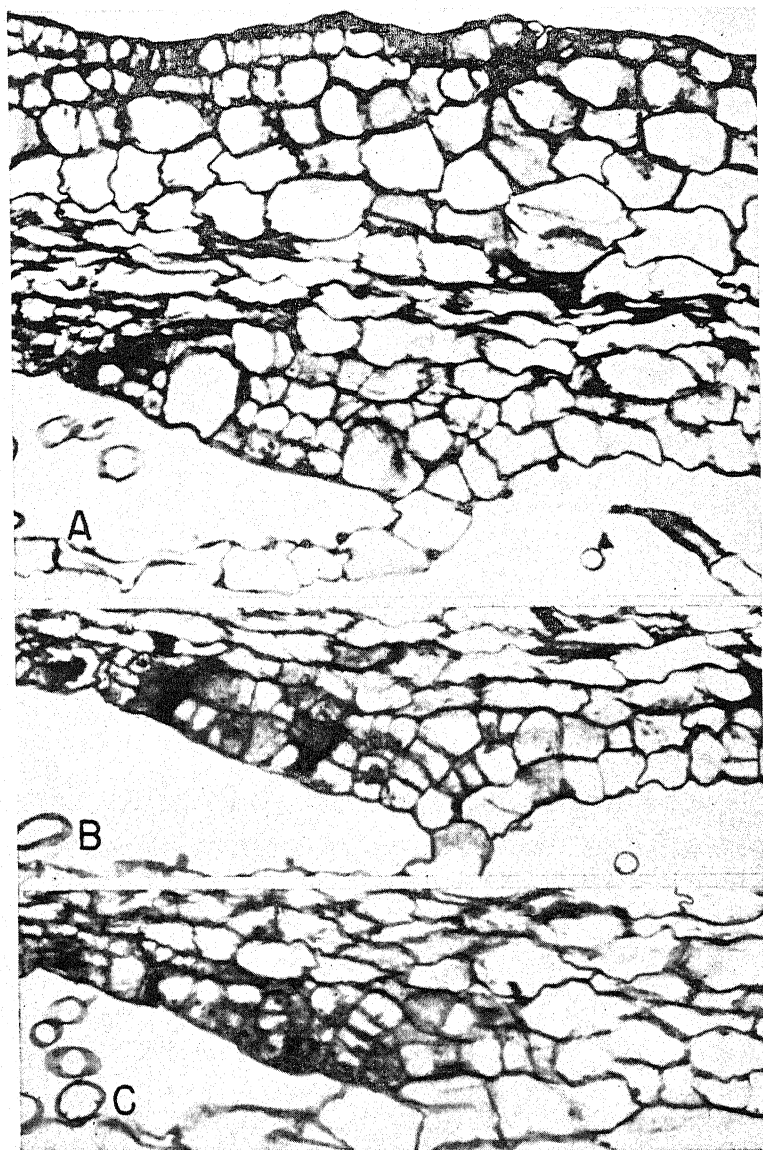


FIG. 11.—Vertical, longitudinal sections of thalli, experiment 8, combination 3, on long photoperiod: *A*, at left, bases of two rhizoids; *B*, similar section. Epidermal and subepidermal cells between bases of rhizoids have become meristematic; *C*, similar section showing more advanced meristematic condition, beginning of regeneration of new thallus.

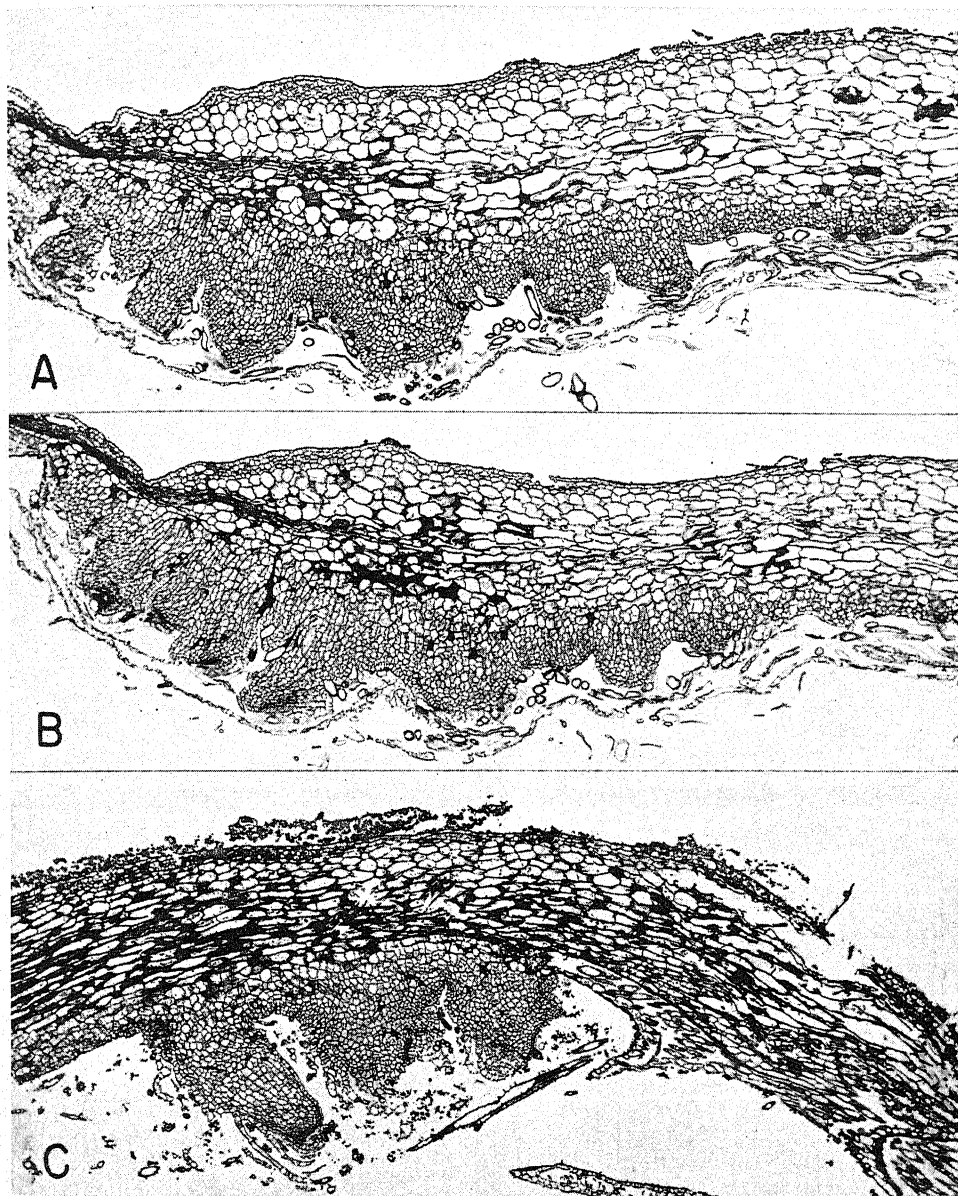


FIG. 12.—Similar to fig. 11, showing progressive stages at *A*, *B*, and *C* of regeneration of thalli

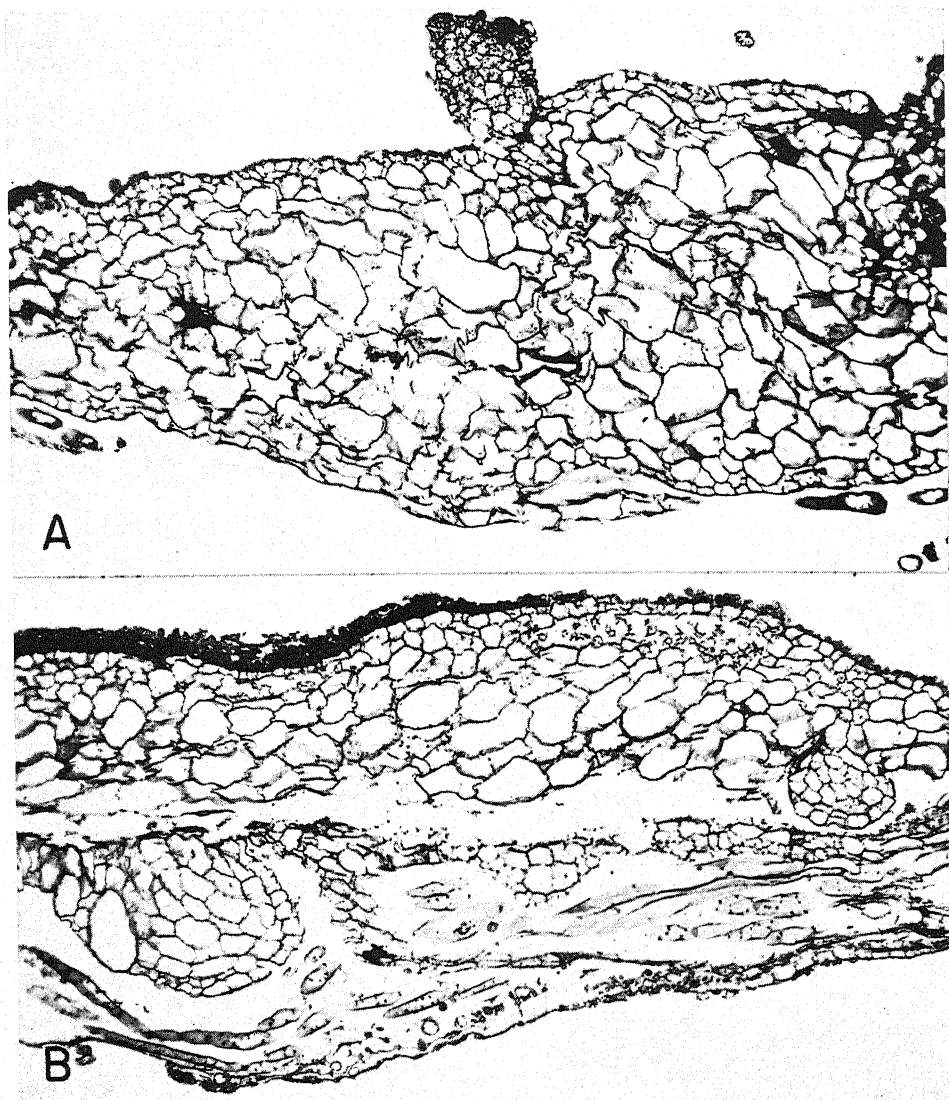


FIG. 13.—*A*, regeneration of young thallus at upper surface of old thallus; *B*, regeneration of thalli from lower surface and from storage cells of old thallus.

in any of the other treatments. The smaller dimensions of the other layers in the storage region, however, reduced the total thickness of the thallus. Gemmae cups in many stages of development were found in nearly all plants sectioned.

#### LACK OF CALCIUM AND REGENERATION OF THALLUS

All combinations in both experiments in which calcium was lacking (1, 3, 6, 10, 15, 21, and 28) regenerated new thalli from the underside of the midrib region. Although such regenerated thalli never became very large, they were visible to the unaided eye. On solutions 1, 3, and 6, numerous thalli were formed; in some cases a regenerated thallus in turn regenerated another one. Fewer thalli were formed on combinations 10, 15, 21, and 28; on the latter, only one of the sectioned plants in experiment 9 possessed a regenerated thallus. Seven- $\mu$  sections of *Marchantia* plants on these combinations presented a series of developmental stages of new thalli (figs. 11-13).

Cells of the lower epidermis on either side of the midrib region, in interrupted longitudinal rows, may differentiate into large, dark-staining cells characteristic of rhizoid initials. These elongate as smooth rhizoids when the apical region has outdistanced them by about twenty cells, although some initials remain dormant for a time. When mature (fig. 9A, B), such rhizoids are smooth walled, grow vertically downward, and attach to the substrate (7, 13). The base of a rhizoid is large, and cells surrounding it usually undergo nuclear and cell divisions but do not enlarge appreciably. The rhizoid base has the appearance of being sunken two or three cell layers into the lower side of the thallus (fig. 11A). In plants grown on solutions lacking calcium, these small epidermal and subepidermal cells surrounding the base of the rhizoid become meristematic (fig. 11B). A small group of cells is formed which protrudes from the lower level of the epidermis (fig. 11C). Initiation of such meristematic groups occurs in basipetal succession (fig. 12A). The meristematic group of cells soon differentiates a mucilage hair (slime papilla), accompanied by a small, acropetally placed depression. In this depression, apical cells are differentiated. Groups of such growing points often occur simultaneously to form a longitudinal row of thalli (fig. 12A, B, C). In median longitudinal section young thalli have the same structure as adult plants, except that meristematic activity is limited to the apical cells and one subepidermal row (fig. 12B, C). The tips of all regenerated thalli are directed toward the tip of the parent plant and may emerge from beneath it. In all plants regenerating ventrally, only the lower epidermis—and at most about five cell layers of the lower part of the thallus—are involved in the production of new thalli.

Two types of exceptions to the method of regeneration just described are shown in figure 13. The uppermost thallus has produced an adventitious bud from the upper epidermis (combination 10, experiment 8). Only one other such example

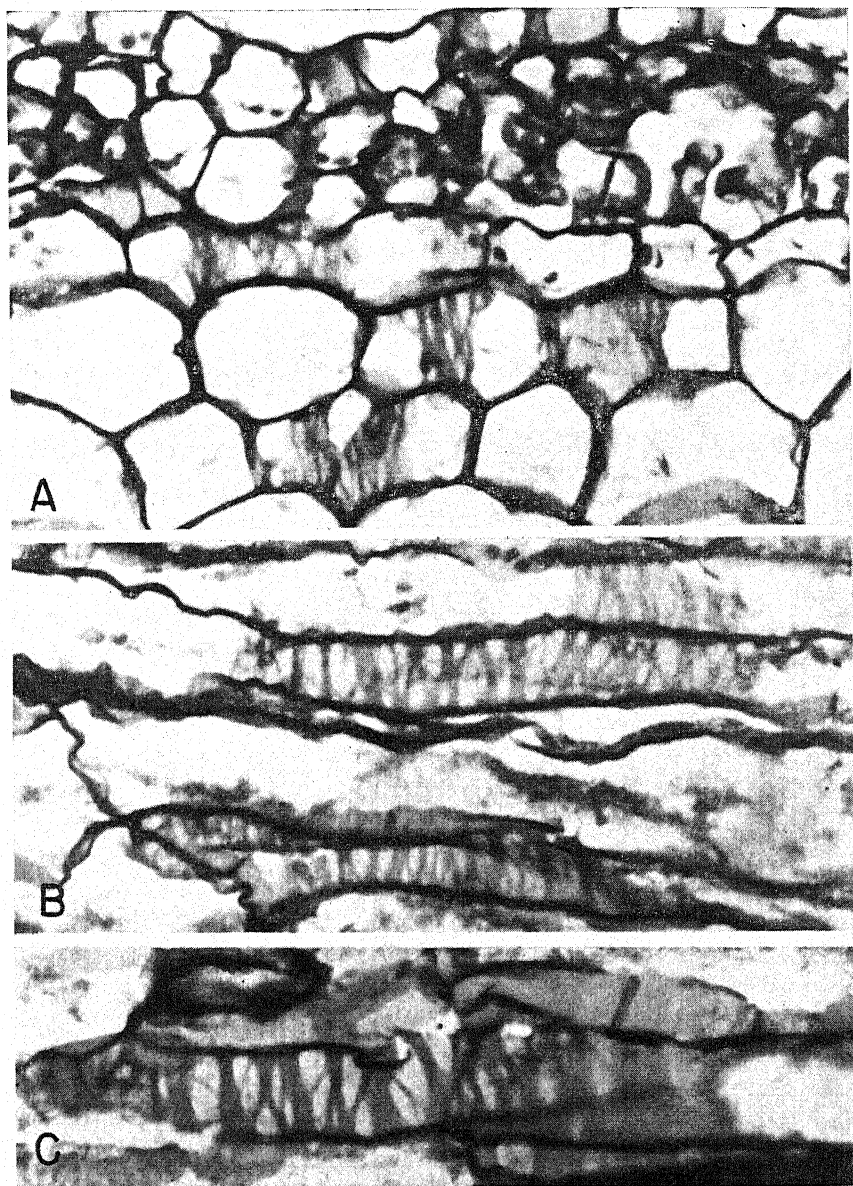


FIG. 14.—Cells of storage region showing wall thickenings as commonly found in all cultures: *A*, transverse section; *B*, *C*, longitudinal sections.

was found (combination 3, experiment 9). In this variation of regeneration only a few upper layers of cells were involved in the production of the new thallus. Because of the absence of air chambers (fig. 13A), it is known that the bud was formed in the midrib region which in this species commonly lacks air chambers.

The second type of exception to the usual method of regeneration was found in one thallus only (combination 3, experiment 9). Necrosis had occurred in a longitudinal layer of the storage tissue, a few cells above the lower epidermis (fig. 13B). A part of this necrotic strand disintegrated, leaving an air space. Storage cells forming the roof of this space became active and formed a small, internal bud. The parent thallus also produced a bud from the lower epidermis in the usual manner (fig. 13B).

KLEIN (20) reports that *Marchantia polymorpha* growing naturally in Hungary regenerated from the furrows of the archegoniophores. Several investigators have shown that in thallus cuttings of thallose liverworts regeneration proceeds from the ventral surface of the apical end (2, 6, 21, 36). VÖCHTING (36) illustrates the younger stages of regeneration in the furrow of the archegoniophore of *Marchantia* following injury. The absence of adventitious bud formation on all nutrient combinations containing only the smallest fraction of the calcium solution indicates that the absence of calcium or the interaction of factors in the absence of calcium may act to stimulate certain ventral cells to become active.

#### STORAGE CELLS WITH WALL THICKENINGS AND PITS

On every nutrient combination and under both photoperiods, *Marchantia* plants possessed elongated cells in the storage region which were thickened on the inside face of their walls. At times these thickenings were very faint and resembled protoplasmic strands. End walls of storage cells of the initial controls possessed scalariform thickenings (fig. 14A). Such thickenings varied in width (fig. 14B, C). These cells were found either near the photosynthetic tissues (fig. 14A), in the middle of the thallus, or very near the lower epidermis (especially on solution 56). They were most prominent in longitudinal sections near the midrib and appear to have been living cells at the time of fixation, since starch grains were present in several such cells (combinations 16 and 11 in experiments 8 and 9, respectively). In necrotic thalli such as those on combinations 10, 15, 21, and 28, these thick-walled cells stained deeply with safranin, which is characteristic of nearly all the nonliving cells. Cells from plants lacking in calcium possessed thickenings which covered most of the wall, leaving only large oval pits.

Storage cells with walls thickened in various ways have been repeatedly mentioned by bryologists for *Marchantia* (3, 7, 12, 18, 26, 35, 37), *Conocephalum* (*Fegatella*) (2, 4, 27), *Lunularia* (32), *Monoclea* (30), *Bryum* and *Mnium* (15), and members of the Dilaenaceae such as *Symphyogyna* (22). Isolated drawings of such

cells are presented by BOLLETER (2), CAVERS (4), and SCHIFFNER (32) for *Conocephalum* and *Lunularia*, respectively. GOLENKIN (14) figures mycorrhizal hyphae inhabiting red-violet storage cells in *Preissia*, *Marchantia palmata* and *M. paleacea*, all of which are said to be pitted in age. The tracheids found by HOLLOWAY (19) in the gametophyte of *Psilotum* probably belong to a category different from those described in the accompanying figures.

### General discussion

While the foregoing results are based upon relatively few plants on each combination of nutrients, some of the data are striking and clear cut. Growth of *Marchantia* is greater on nutrient solutions with a low osmotic concentration than on those of a relatively high one. The effect of length of photoperiod is also marked; vegetative extension, gametangiophore production, and vegetative activity are greater under long photoperiod than short photoperiod, although under the latter condition formation of gemmae cups is increased. This seems true regardless of the nutrient solution upon which the plants are grown. In our experience it has also been found that the particular strains of *M. polymorpha* used will grow very rapidly, even under conditions of relatively high light intensity and relatively high greenhouse temperatures, dependent upon the composition of the nutrient solution supplied. It has been found repeatedly in different experiments that plants growing on the various nutrient solutions produced much greater growth than did comparable plants growing on garden loam soil, even though the plants on such soil were abundantly supplied with water. On some of the nutrient combinations the plants grew and gained more dry weight than did plants observed under field conditions at comparable seasons of the year. The only plants which have been observed growing as rapidly and as large in natural habitats were found on areas from which vegetation had recently been burned or on the charcoal and ash residues of burned trash piles.

The marked effects resulting from variations in the proportions of the major cations and anions indicate clearly that *Marchantia* produces much greater growth on nutrient solutions relatively low in phosphorus content, regardless of great variation in sulphate and nitrate content. It is likely that with certain combinations even greater growth may be produced. If, for example, larger triangles were used, so that the variations were not so great from one combination to another, and if the number of plants grown on each particular solution were greater, a more rigorous statistical analysis of the results could be made. It is highly desirable to establish cation triangles in which much lower concentrations of phosphorus are present, and it would be equally desirable to test higher concentrations of nitrate. Under such range of combinations in the cation triangle, it might well be that small differences in the content of potassium, calcium, or magnesium would show



even more striking effects than were obtained in the present experiments. The method used is applicable to the general study of problems of mineral nutrition.

### Summary

1. *Marchantia* grows readily on glass cloth in an open moist chamber when supplied with an appropriate nutrient solution. A method for growing it under controlled conditions of nutrient supply and photoperiod is described. A male and a female strain of *Marchantia polymorpha* were employed, each strain propagated vegetatively from a stock grown from a single plant.

2. A range of fifty-six nutrient solutions, each having an osmotic concentration of approximately 0.285 atmospheres, was used. The cations involved were K, Ca, and Mg, and the anions  $\text{NO}_3$ ,  $\text{PO}_4$ , and  $\text{SO}_4$ .

3. Cultures containing the cations K and Mg but without Ca all regenerate new thalli, formed in the main from adventitious buds arising from cells on the ventral side of the thallus at the midrib region. In cultures lacking  $\text{NO}_3$  and  $\text{PO}_4$  the ventral layers of cells develop a red-violet color in the walls. The absence of Mg and  $\text{SO}_4$  does not result in so striking a response as lack of the other ions. Phosphate is required in very small quantities. Up to the limits of the quantities supplied, plants make greater growth with increasing amounts of  $\text{NO}_3$ , provided all other ions are present.

4. Some of the plants were grown on long photoperiod, 18 hours of light out of each 24-hour cycle, and on short photoperiod, 9 hours of light out of each 24. The differences between the lots of plants grown on the two photoperiods were both quantitative and qualitative. Irrespective of the range of nutrient supply, plants on long photoperiod were larger and had greater dry weight than similar plants on comparable solutions on short photoperiod. Irrespective of the range of nutrient supply, plants on short photoperiod produced a greater total number of gemmae cups but on long photoperiod a greater number of gametangiophores than comparable plants on short photoperiod.

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# CHEMICAL AND HISTOLOGICAL RESPONSES OF BEAN PLANTS GROWN AT DIFFERENT LEVELS OF NUTRITION TO INDOLEACETIC ACID<sup>1</sup>

ORA SMITH, L. B. NASH, AND G. E. DAVIS

(WITH ELEVEN FIGURES)

## Introduction

Considerable histological work has recently been published on the responses of various plants, including the bean (3, 4, 5, 6), to applications of indoleacetic acid and other growth substances. Other investigations (1, 7, 8, 9, 10) have shown that application of indoleacetic acid to cuttings or decapitated plants affects their metabolism and rate of transfer of carbohydrate and nitrogenous compounds. The writers, however, know of no investigation wherein the histological studies and chemical analyses were made on the same material grown under the same conditions at the same time. It has not yet been shown whether roots are formed after translocation of certain materials, probably as a result of it, or whether the transfer of carbohydrate and nitrogenous materials occurs after root primordia have been stimulated more directly by application of the growth substance. The present paper relates certain histological changes to chemical changes of the same lots of plants grown under the same conditions.

There is little published work (3) on the effects of various levels of nutrition of the plants on their response to the growth substance application. Obviously differences in nutrition may greatly alter this response. Again, most studies have involved plants from which the roots or the terminal growing point were removed before treatment. Only the cotyledons were removed from the seedlings used in these studies.

## Material and methods

**CHEMICAL STUDIES.**—Seedlings of kidney bean, *Phaseolus vulgaris*, variety Pencil Pod Black Wax, were grown under two levels of nutrition. One level secured seedlings high in nitrogen and low in carbohydrates, and the other level secured seedlings low in nitrogen and high in carbohydrates. On July 21, about 2500 seedlings of each level of nutrition were started in washed quartz sand. Flats 4×3 feet were used, and all seedlings were grown in the greenhouse.

The high nitrogen, low carbohydrate seedlings were grown as follows: complete nutrient solution was added each day, and the sand was kept sufficiently moist

<sup>1</sup> Paper no. 213, Department of Vegetable Crops, Cornell University, Ithaca, New York. Indoleacetic acid supplied by Merck & Co. and Eastman Kodak Co.

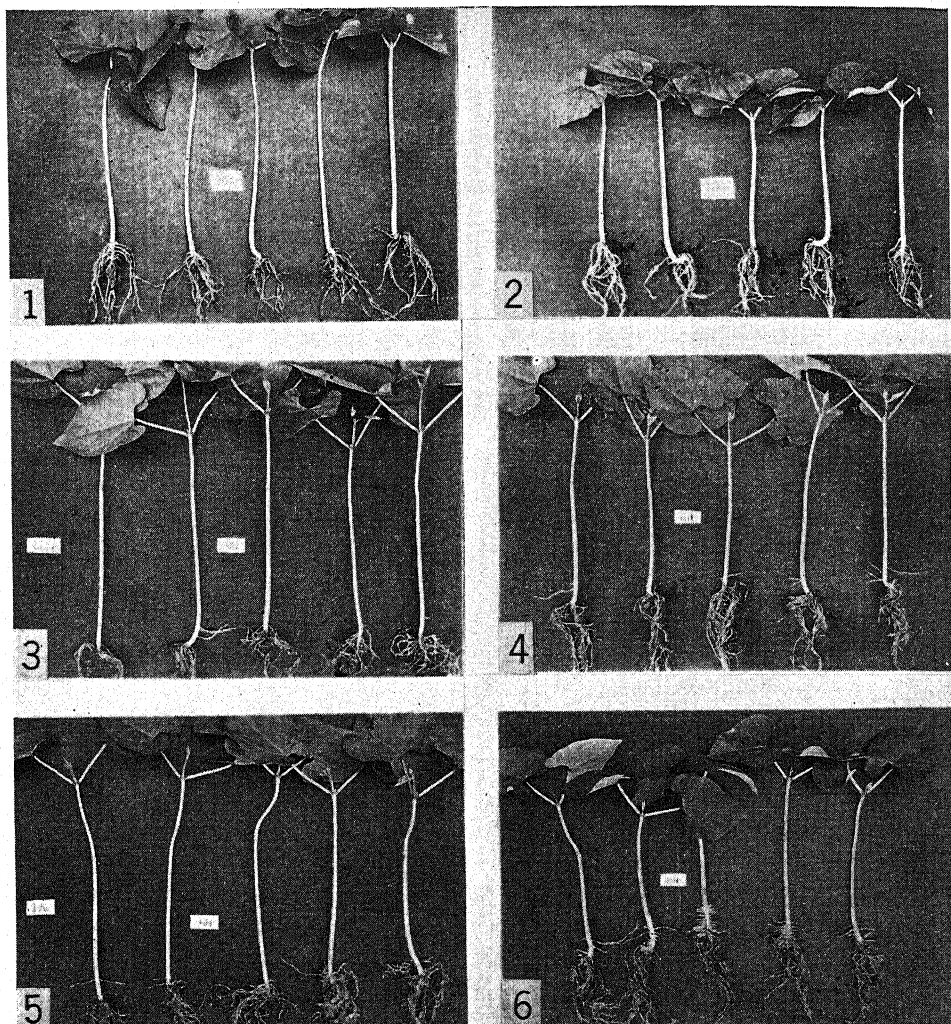
for continued growth. During the 2 days previous to treatment the seedlings were shaded by brown wrapping paper placed about 6 inches above them. The low nitrogen, high carbohydrate seedlings were grown as follows: nutrient solution containing nitrogen was added in sufficient quantity at the time of planting to sprout the beans, but after the first addition only enough nitrogen-free solution was added each day to keep the seedlings from wilting. The sand was kept dry. As a result growth was checked and carbohydrates accumulated. These seedlings were also given 4 hours of artificial illumination, each of the two nights previous to treatment. Two 100-watt bulbs were used above each flat.

As was expected, the high nitrogen, low carbohydrate seedlings were tall, spindly, and soft as compared with the shorter, stockier, and hardier low nitrogen, high carbohydrate seedlings (figs. 1, 2).

Eight days after planting, when the second internode was about 1 cm. in length, the seedlings were prepared for treatment. The cotyledons were removed and all seedlings were taken from the flats. The seedlings, carefully selected for uniformity, were treated by standing in bunches of about 100 in a 0.01 per cent aqueous solution of indoleacetic acid or in tap water, placed so that all the roots and about 1 inch of the lower hypocotyls were submerged. The indoleacetic acid was first dissolved in a small quantity of ethyl alcohol. Duration of the treatment was 4 hours. After treatment, the seedlings were washed with tap water and transplanted into washed quartz sand in large greenhouse flats. All seedlings were then watered with complete nutrient solution and otherwise treated the same. For the first 2 days after treatment, the seedlings were kept in the headhouse and then removed to benches in the greenhouse, where the light had been previously reduced by a coat of whitewash on the glass. The temperature ranged from 70° to 100° F. The benches and floor of the house were kept moist enough so that the plants did not wilt badly during the middle of the day.

Samples of 100 representative seedlings from each of the treatments were taken between 9 A.M. and 10 A.M. and sectioned into roots, lower and upper half of hypocotyl, first internode, and leaves. The leaf sample included the petioles. The dry weight of these sections was determined by placing in a forced draft oven at 85° C. for 48 hours and weighing. Total nitrogen to include nitrates and total sugars were determined by methods given by the A.O.A.C. (2). Alcohol-insoluble acid-hydrolyzable carbohydrates were determined on the residue from the sugar extraction and were calculated as starch.

**HISTOLOGICAL STUDIES.**—Samples for histological observation were taken from about  $\frac{1}{2}$  inch of the lower end of the hypocotyl, since this is the region where new roots are expected to appear. These sections were fixed in a modified chromoacetic acid mixture, imbedded in paraffin, and cut 15–20  $\mu$  in thickness. Both longitudinal and cross sections of the hypocotyl were made in order to observe as



FIGS. 1-6.—Fig. 1, high nitrogen, low carbohydrate seedlings at time of treatment with indoleacetic acid solution 8 days after planting. Fig. 2, low nitrogen, high carbohydrate seedlings 8 days after planting. Figs. 3-6, plants 6 days after experiment was started: 3, high nitrogen, low carbohydrate seedlings not treated. 4, low nitrogen, high carbohydrate seedlings not treated. 5, high nitrogen, low carbohydrate seedlings treated with 0.01 per cent acid. 6, low nitrogen, high carbohydrate seedlings, treated.

much of the tissue as possible. The tissue was stained with safranin and counter-stained with crystal violet in clove oil.

The preceding samples were taken from all treatments at 12-hour intervals up to 48 hours; thereafter at 72, 96, 144, and 192 hours and 8, 11, and 14 days.

## Experimental results

### GROWTH RESPONSES

Treatment of seedlings with indoleacetic acid influenced subsequent growth at both levels of nutrition. Treatment increased the production of roots, suppressed elongation of the first internode, and delayed development of the second internode. At both levels of nutrition the suppression of top growth in the treated seedlings was about equally marked; but great difference was found in the time of production of new roots (table 1, figs. 3-6).

TABLE 1

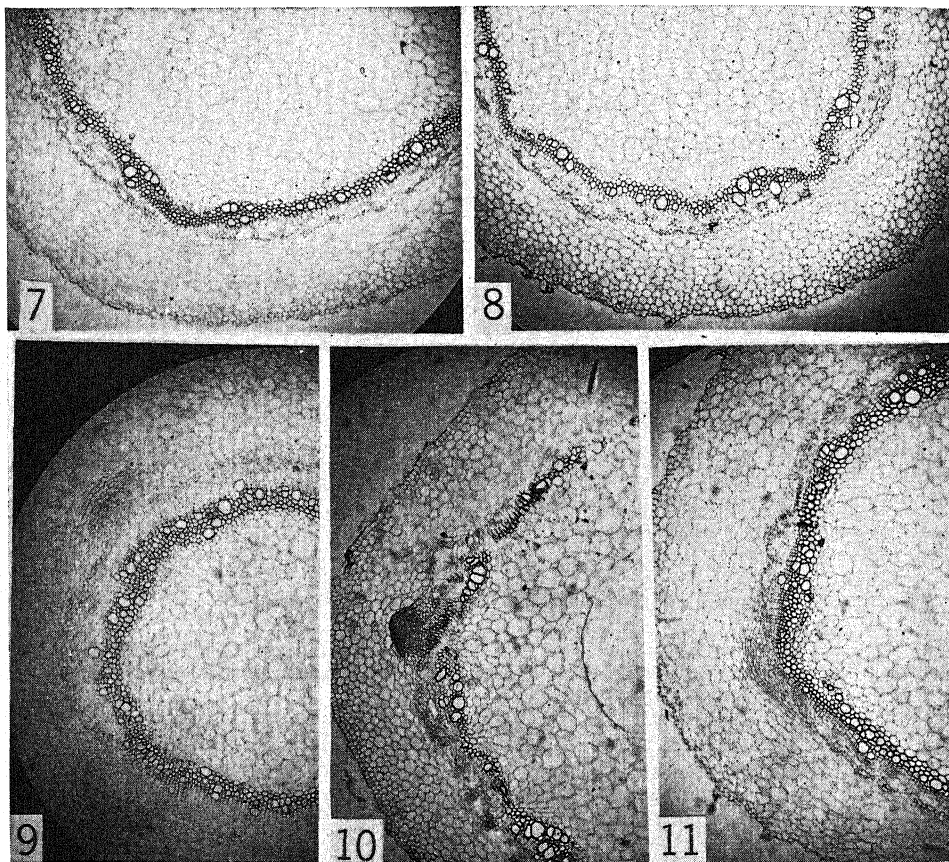
TIME OF APPEARANCE OF ROOT PRIMORDIA AND VISIBLE ROOTS FROM HYPOCOTYL

TREATMENT	APPEARANCE OF FIRST ROOT PRIMORDIA IN HYPOCOTYL	APPEARANCE OF NEW ROOTS OUTSIDE HYPOCOTYL
1. Low nitrogen, high carbohydrate, treated.....	36 hours	48 hours
2. Low nitrogen, high carbohydrate, not treated.....	72 hours	96 hours
3. High nitrogen, low carbohydrate, treated.....	144 hours	192 hours
4. High nitrogen, low carbohydrate, not treated.....	None after 268 hours	None after 268 hours

Cross sections of the lower hypocotyls of untreated plants at the start of the experiment are shown in figures 7 and 8.

Under conditions of low nitrogen, high carbohydrates, and treated with indoleacetic acid, root primordia were observed scattered throughout the hypocotyl 36 hours after treatment. These primordia apparently originate from the phloem tissue, above the intervacular area, and are frequently found on the same level at four diagonally opposite points on the hypocotyl (fig. 9). In plants of this lot new primordia continued to be laid down through the sixth day after treatment. These new primordia could be observed together with the more developed secondary roots which had been initiated earlier. Apparently new root initiation stopped soon after the sixth day, as none was observed in the samples taken later. In plants low in nitrogen and high in carbohydrates, new root primordia were found at 72 hours without treatment with indoleacetic acid, but not in the same abundance as in those treated with the acid (fig. 10). A few primordia were found again at 6 days and none thereafter.

Treating the high nitrogen, low carbohydrate plants with indoleacetic acid stimulated root primordia to appear by the sixth day after treatment (fig. 11).



FIGS. 7-11.—Figs. 7, 8, cross sections of lower hypocotyls of seedlings at beginning of experiment: 7, low nitrogen, high carbohydrate. 8, high nitrogen, low carbohydrate. Figs. 9-11, cross sections of lower hypocotyls showing root initials: 9, treated, low nitrogen, high carbohydrate seedlings, 36 hours after treatment. 10, low nitrogen, high carbohydrate seedlings, untreated, 72 hours after treatment. 11, treated, high nitrogen, low carbohydrate seedlings, 6 days after treatment.

Under the same conditions but without treatment no primordia could be found in any of the samples through the eighteenth day after treatment, when the experiment was terminated.

Treated plants produced more roots than did the untreated plants. After treatment, however, roots appeared much sooner and in greater numbers in the low nitrogen, high carbohydrate plants than in the high nitrogen, low carbohydrate plants. This was true for induced roots on the lower hypocotyl as well as for new secondary roots on the pre-existing root systems.

Conditions of low nitrogen or high carbohydrates are conducive to the production of new roots on the lower hypocotyl without indoleacetic acid treatment, although roots appear in not nearly the same quantity nor so early as when treated. Since no root primordia could be observed under conditions of high nitrogen, low carbohydrates, without treatment, it follows that in these experiments the formation of new root primordia was largely influenced by the carbohydrate reserves in the plant.

#### INFLUENCE OF INDOLEACETIC ACID ON DISTRIBUTION OF DRY WEIGHT

The seedlings were kept for the first 2 days after treatment in the headhouse, where the light intensity was low and the temperature relatively high. The greenhouse to which the seedlings were transferred had previously been shaded by a coat of whitewash on the glass. Consequently there was little actual gain in dry weight the first few days after treatment.

The data of table 2 show that there were differences in distribution of materials in the seedlings. As already pointed out, treating the low nitrogen, high carbohydrate seedlings with indoleacetic acid markedly increased the number of roots, and table 2 shows also an increase in the dry weight of the roots. However, treatment of the high nitrogen, low carbohydrate seedlings resulted in little or no consistent increase in dry weight of the roots above those untreated. The dry weight of the lower hypocotyls increased in all treatments for the duration of the experiment, but after 4 days the dry weights of lower hypocotyls of the untreated seedlings were greater with one exception than the treated. The probable reason for this greater increase after the first 4 days is that material in this region was not translocated into the roots as fast as from the treated lower hypocotyls. In the treated, low nitrogen, high carbohydrate seedlings the plants had a lower dry weight of the first internodes at every sampling and also of the upper hypocotyls after the first sampling than the untreated ones.

In the high nitrogen, low carbohydrate seedlings it was not until 8 days or more after treatment that the dry weights of the upper hypocotyls and first internodes



of treated seedlings were appreciably and consistently lower than of the untreated seedlings.

This would indicate that the treated, high nitrogen, low carbohydrate seedlings were less responsive to changes in the distribution of dry weight than were the treated, low nitrogen, high carbohydrate seedlings.

TABLE 2

DRY WEIGHT (IN GRAMS) OF 100 KIDNEY BEAN SEEDLINGS TREATED WITH WATER AND WITH 0.01 PER CENT AQUEOUS SOLUTION OF INDOLEACETIC ACID

DAYS AFTER TREATMENT	ROOTS		LOWER HYPOCOTYLS		UPPER HYPOCOTYLS		FIRST INTERNODES		LEAVES	
	WATER	ACID	WATER	ACID	WATER	ACID	WATER	ACID	WATER	ACID
HIGH NITROGEN, LOW CARBOHYDRATE										
0.....	2.028	.....	2.024	.....	1.521	.....	1.155	.....	6.782	.....
2.....	2.173	2.296	2.592	2.184	1.983	1.641	1.437	1.297	7.693	7.255
4.....	2.200	2.221	2.448	2.373	1.882	1.923	1.566	1.528	7.988	9.399
6.....	1.992	2.613	2.596	2.634	1.956	2.290	1.549	1.691	8.653	10.249
8.....	3.234	3.672	2.829	2.562	2.192	2.050	1.732	1.807	9.315	10.877
11.....	2.889	2.939	3.523	3.029	2.775	2.204	2.333	2.056	11.926	12.441
14.....	5.079	4.030	3.773	3.251	2.948	2.478	2.537	2.257	12.644	12.301
LOW NITROGEN, HIGH CARBOHYDRATE										
0.....	3.521	.....	1.845	.....	1.725	.....	0.866	.....	7.126	.....
2.....	1.983	2.813	1.980	2.141	1.808	1.833	0.971	0.939	6.854	7.100
4.....	1.869	2.618	2.082	2.164	1.871	1.852	1.306	1.013	7.505	7.608
6.....	3.483	4.053	2.771	2.407	2.210	1.960	1.521	1.174	9.481	9.268
8.....	3.872	5.224	2.627	2.196	2.137	1.854	1.556	1.292	9.132	10.106
11.....	4.864	6.387	2.908	2.882	2.454	2.362	2.047	2.006	11.762	14.502
14.....	6.926	9.453	3.999	3.664	3.167	2.864	2.653	2.554	26.812	29.586

#### INFLUENCE OF INDOLEACETIC ACID ON TOTAL NITROGEN DISTRIBUTION

In general, the results presented in table 3 indicate that treatment with indoleacetic acid resulted in greater transfer of nitrogen from the upper parts of the seedlings, particularly from the leaves, into the lower hypocotyl and roots. The treated roots and lower hypocotyls are, with few exceptions, higher at each sampling than the roots and lower hypocotyls of the untreated seedlings; however, the differences in total nitrogen between the treated and untreated roots at every stage of sampling except the first is greater in the high nitrogen, low carbohydrate seedlings than in the low nitrogen, high carbohydrate seedlings.

During the first 8-11 days after treatment, the upper hypocotyl, first inter-

nodes, and leaves of the treated, high nitrogen, low carbohydrate seedlings showed greater percentage loss of total nitrogen than did the same parts of untreated seedlings. At 11 or 14 days, however, the percentage of total nitrogen in these parts was higher in the treated seedlings than in the untreated. In the low nitrogen, high carbohydrate seedlings the movement of nitrogen from the upper portions of the treated seedlings occurred to a lesser degree than in the treated, high nitrogen, low carbohydrate seedlings. In fact, in most cases the percentage of nitrogen in the upper hypocotyl and first internodes of the treated, low nitrogen,

TABLE 3

PERCENTAGE TOTAL NITROGEN CONTENT OF KIDNEY BEAN SEEDLINGS TREATED WITH WATER AND WITH 0.01 PER CENT AQUEOUS SOLUTION OF INDOLEACETIC ACID

DAYS AFTER TREATMENT	ROOTS		LOWER HYPOCOTYL		UPPER HYPOCOTYL		FIRST INTERNODES		LEAVES	
	WATER	ACID	WATER	ACID	WATER	ACID	WATER	ACID	WATER	ACID
HIGH NITROGEN, LOW CARBOHYDRATE										
0.....	5.07	.....	6.72	.....	6.42	.....	6.42	.....	7.62	.....
2.....	2.72	2.97	6.60	6.17	6.65	6.62	6.25	6.15	7.27	6.75
4.....	3.22	3.90	6.20	6.20	6.47	6.35	6.35	6.30	6.80	6.20
6.....	3.17	4.10	6.00	5.90	5.87	5.67	5.75	5.65	6.35	5.62
8.....	2.55	3.02	5.52	5.82	5.42	5.35	5.55	5.17	6.40	5.85
11.....	2.87	3.77	4.00	5.00	3.60	4.65	3.82	4.45	5.52	5.10
14.....	2.32	3.37	3.12	5.50	2.70	4.87	2.75	4.67	4.90	5.32
LOW NITROGEN, HIGH CARBOHYDRATE										
0.....	3.27	.....	5.00	.....	4.87	.....	4.70	.....	6.27	.....
2.....	3.20	3.57	2.85	5.80	5.15	5.67	4.95	4.92	6.85	6.65
4.....	3.40	4.02	5.10	5.85	4.52	5.52	4.80	4.35	6.85	6.32
6.....	2.50	3.22	3.70	5.00	3.30	4.67	3.65	4.70	5.82	5.70
8.....	2.50	2.72	3.15	4.90	2.90	4.52	3.25	4.35	5.22	5.27
11.....	2.22	1.90	2.32	3.22	2.00	3.02	2.22	2.87	4.35	4.60
14.....	1.87	2.05	1.45	3.15	1.20	2.40	1.50	2.40	4.50	4.02

high carbohydrate seedlings was higher than that in the same parts of the untreated seedlings. The results indicate that the effect of treatment on the distribution of total nitrogen is affected by the level of nitrogen nutrition.

#### INFLUENCE OF INDOLEACETIC ACID ON DISTRIBUTION OF CARBOHYDRATES

At almost every period of sampling the total sugar (table 4) is lower in all parts of the treated seedlings than in the same portions of untreated seedlings. One of the probable reasons for the decreased total sugar content of the roots

and lower hypocotyls of the treated seedlings may be that the sugars in these regions were being more rapidly converted into proteins and other insoluble compounds, owing to the increased rate of root development. Likewise, the decreased total sugar in the upper parts of the treated plants may be due to the transfer of sugars out of this region into the lower parts of the seedlings.

TABLE 4

PERCENTAGE TOTAL SUGARS OF KIDNEY BEAN SEEDLINGS TREATED WITH WATER AND WITH 0.01 PER CENT AQUEOUS SOLUTION OF INDOLEACETIC ACID

DAYS AFTER TREATMENT	ROOTS		LOWER HYPOCOTYL		UPPER HYPOCOTYL		FIRST INTERNODES		LEAVES	
	WATER	ACID	WATER	ACID	WATER	ACID	WATER	ACID	WATER	ACID
HIGH NITROGEN, LOW CARBOHYDRATE										
0.....	None	.....	0.08	.....	0.40	.....	2.07	.....	0.08	.....
2.....	None	None	0.16	0.12	None	None	None	None	0.04	0.04
4.....	1.07	0.44	0.24	0.16	None	None	1.90	0.40	0.08	0.56
6.....	1.63	0.24	3.77	0.24	None	None	2.50	0.95	1.80	0.74
8.....	1.99	0.80	3.19	0.24	0.72	0.24	3.20	3.98	1.70	1.11
11.....	1.60	0.48	5.81	1.95	1.23	0.52	6.23	3.24	2.59	1.19
14.....	1.98	1.75	4.68	0.92	5.22	0.95	5.10	3.71	2.25	1.39
LOW NITROGEN, HIGH CARBOHYDRATE										
0.....	0.76	.....	0.64	.....	1.91	.....	5.40	.....	1.01	.....
2.....	None	None	0.16	0.20	None	None	0.88	0.32	0.04	0.04
4.....	2.69	1.23	3.16	0.24	None	None	4.30	0.32	0.91	0.16
6.....	1.91	0.24	5.57	0.28	2.07	0.24	4.76	4.11	4.12	1.39
8.....	2.15	1.11	2.15	0.24	1.23	0.30	4.33	7.37	1.27	1.17
11.....	1.19	0.40	3.24	1.63	4.82	4.45	7.84	4.78	2.57	1.80
14.....	3.06	0.80	5.77	3.02	6.74	4.41	7.84	4.92	2.44	2.03

The lower and upper hypocotyls of treated seedlings of both levels of nutrition showed a greater percentage of alcohol-insoluble acid-hydrolyzable carbohydrates 2 days after treatment than did the same parts of untreated seedlings (table 5). In most other instances, however, after this period the percentage of alcohol-insoluble acid-hydrolyzable carbohydrates was higher in all parts of the untreated seedlings than of the treated, except in the leaves of the low nitrogen, high carbohydrate seedlings. The reason that the percentage of this carbohydrate fraction is usually higher in all parts of the untreated seedlings than in the treated probably is the same as given for the higher total sugars in all parts of the untreated seedlings.

TABLE 5

PERCENTAGE ALCOHOL-INSOLUBLE ACID-HYDROLYZABLE CARBOHYDRATES OF KIDNEY BEAN SEEDLINGS TREATED WITH WATER AND 0.01 PER CENT AQUEOUS SOLUTION OF INDOLEACETIC ACID

DAYS AFTER TREATMENT	LOWER HYPOCOTYL		UPPER HYPOCOTYL		FIRST INTERNODES		LEAVES	
	WATER	ACID	WATER	ACID	WATER	ACID	WATER	ACID
HIGH NITROGEN, LOW CARBOHYDRATE								
0.....	5.03	.....	8.92	.....	22.10	.....	5.88	.....
2.....	3.54	4.32	9.91	11.05	17.85	25.77	8.57	4.62
4.....	11.19	10.20	11.47	10.41	19.68	17.07	9.05	7.84
6.....	5.03	7.58	12.32	12.11	17.56	18.83	8.64	8.14
8.....	12.79	13.32	16.14	15.36	16.85	18.76	7.42	7.22
11.....	19.61	14.99	22.09	13.10	24.42	13.86	10.13	7.65
14.....	.....	.....	27.75	15.10	21.81	14.58	9.67	7.82
LOW NITROGEN, HIGH CARBOHYDRATE								
0.....	8.99	.....	13.88	.....	23.37	.....	9.42	.....
2.....	3.89	4.11	14.66	16.28	31.01	22.23	6.48	7.12
4.....	3.82	2.97	13.17	12.11	17.29	27.33	7.42	7.65
6.....	16.57	9.70	19.26	16.29	21.09	21.58	9.03	6.23
8.....	20.18	15.10	20.25	15.22	16.55	16.24	7.65	7.26
11.....	23.08	14.16	26.06	16.85	22.30	21.10	9.37	10.41
14.....	24.17	15.20	35.82	22.75	24.22	20.04	15.89	10.48

### Summary

1. One lot of kidney bean seedlings was grown under conditions of high nitrogen and low carbohydrate; another lot with low nitrogen and high carbohydrate. After harvesting, dry weight, total nitrogen to include nitrates, total sugars, and alcohol-insoluble acid-hydrolyzable carbohydrates determinations were made.

2. Treatment with indoleacetic acid greatly increased the rate of root formation in the low nitrogen, high carbohydrate seedlings. The response of the high nitrogen, low carbohydrate seedlings to treatment was much slower. Apparently the carbohydrates were the limiting factor in the production of new roots after treating the high nitrogen, low carbohydrate seedlings with the acid.

3. The high nitrogen, low carbohydrate seedlings were less responsive to changes in distribution of dry weight. This lack of or slow response to was probably due to the low supply of available carbohydrates which could be translocated to the region of treatment.

4. In general, treatment with indoleacetic acid was accompanied by greater

transfer of nitrogen from the upper part of the seedlings to the lower parts than in the untreated seedlings. The movement of nitrogen following treatment was greater from leaves to roots and lower hypocotyl in the high nitrogen, low carbohydrate seedlings than in those low in nitrogen and high in carbohydrates.

5. Treatment decreased the percentage of total sugars in all parts of the seedlings. The percentage total sugars in all parts of the treated seedlings at both levels of nutrition was still lower at the end of the experiment than in those seedlings not treated with the growth substance.

6. In general, the percentage alcohol-insoluble acid-hydrolyzable carbohydrates increased in the hypocotyl and to a lesser extent in the leaves with the age of the seedling and to a greater degree in untreated plants than in those which had been treated. The percentage of hydrolyzable carbohydrates in the first internode of untreated seedlings was approximately the same at the end of the experiment as at the beginning, whereas internodes of treated seedlings decreased in percentage of this constituent.

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## METHOD FOR COVERING EMASCULATED FLOWERS IN PLANT BREEDING

J. R. KING

(WITH FOUR FIGURES)

Plant breeders have always been concerned with the problem of properly covering emasculated blossoms as a protection against contamination by insect visits. Because of the many difficulties experienced with paper bags and similar covers, an effort was made to secure a covering that would eliminate some of these disadvantages. Such a cover should be easy to put over a branch and should be insect proof. It must be resistant to wind and rain and also must permit pollen germination beneath it and air circulation through it.

In the development of an idea for making such a cover, Warner Bros. Pictures co-operated in the spring of 1938 by demonstrating to me and then lending me a "mechanical spider." Later a small and modified model of this machine was built at Davis, California.

The web-spinning machine (fig. 1) works on the principle of centrifugal force. A specially designed centrifuge cup is mounted on the drive shaft of a fractional horse-power electric motor and in front of a fan. Rubber cement is used to make the webs. The cement is forced out by centrifugal force through the two lips of the whirling cup and is driven by a fan in the form of a revolving thin mass, which quickly spins itself into a web wherever it comes into contact with an object.

The web may be made as thick as desired and can be spun over any part or over all of a large branch (fig. 2). It is applied quickly and easily and does not damage the pistils, even when torn off. Pollen will germinate beneath the cements and cement mixtures tested, and the webs are insect proof when carefully and evenly spun (figs. 3, 4).

In the spinning process, the small strands of rubber cement are actually laid over the flower parts, so that there is no detectable damage from abrasive action such as may occur when a bag is put over a branch. There is no stretching or pulling during the spinning. The breeze created by the electric-fan blades will bend many outside pistils so that the rubber strands strike and accumulate on the styles and ovaries while the stigmas are untouched and point away from the web. This gives added protection from insects that may alight on the web. The web may be made more wind-and-rain resistant by quickly wrapping loose or flowing rubber strands around the body of the web or by pressing them against it (fig. 2). As the web is, of course, less easy to remove than a paper bag or similar cover, the more

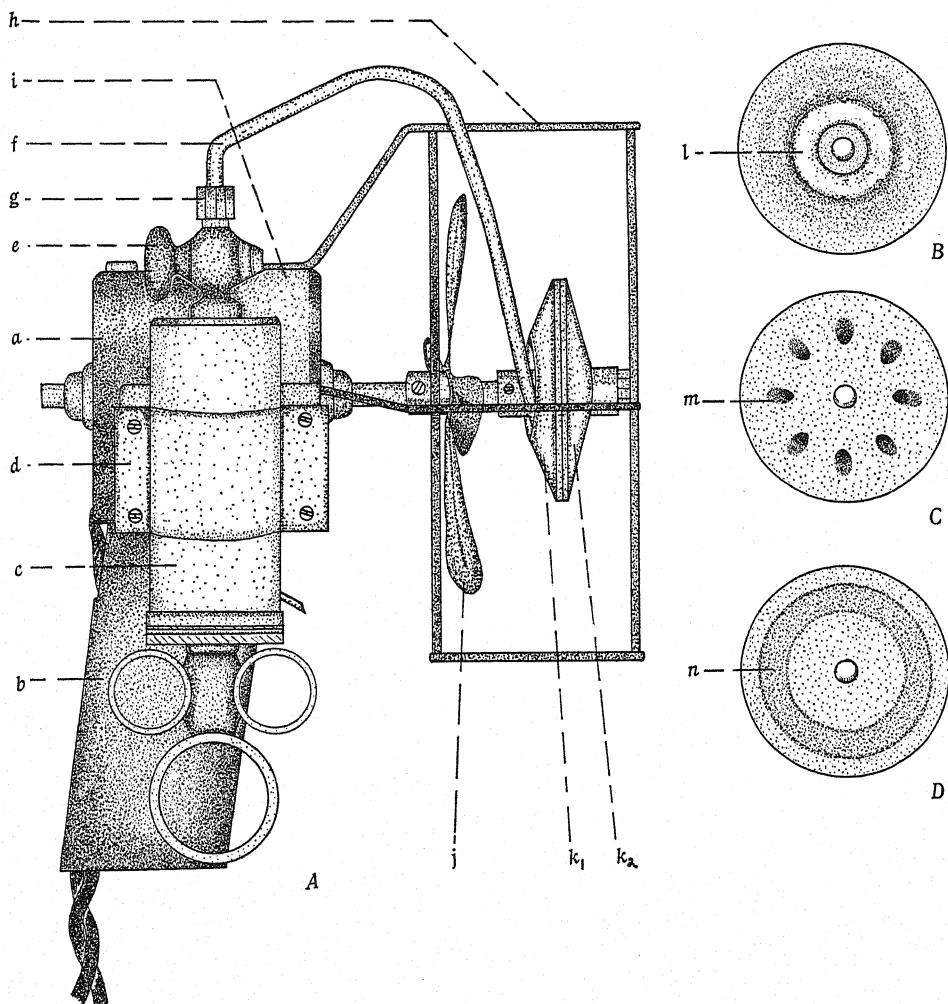
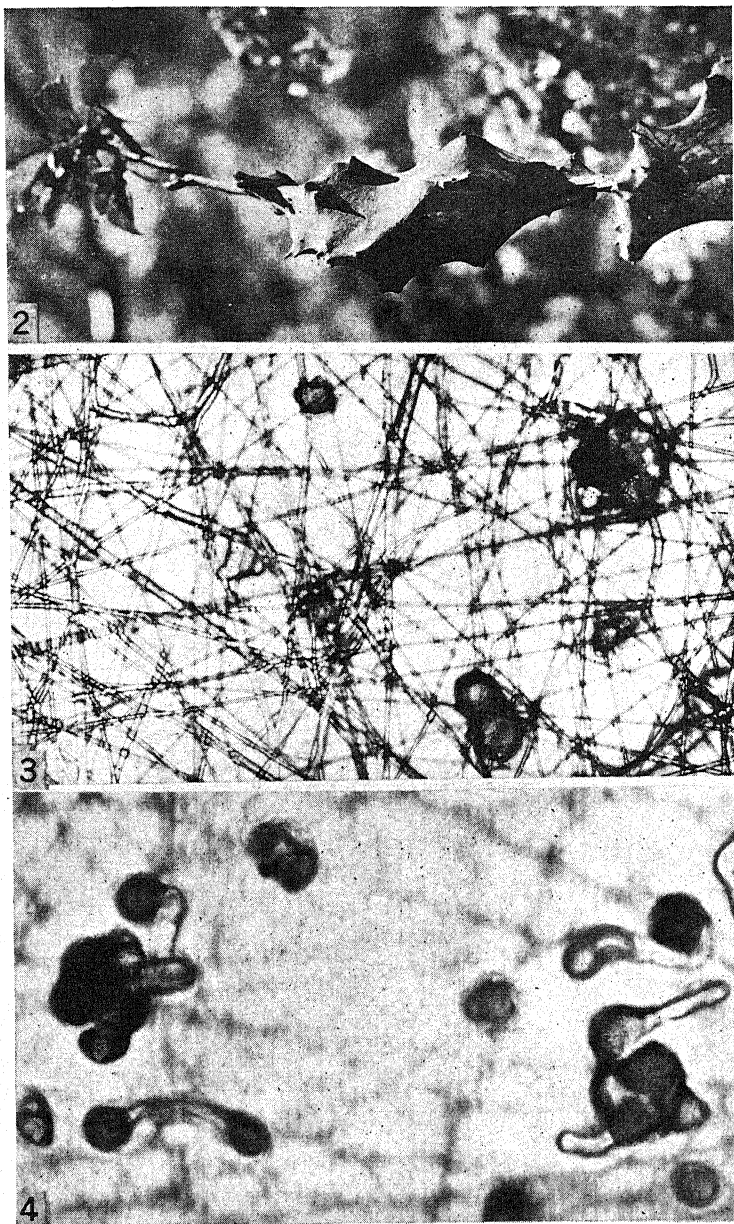


FIG. 1.—*A*, machine used in spinning rubber cement webs over emasculated flowers in plant breeding: (*a*) fractional HP electric motor with pistol grip (*b*); (*c*) metal syringe containing rubber cement; (*d*) metal strap fastening syringe to motor; (*e*) stopcock regulating flow of cement through metal tube (*f*); (*g*) compression fitting connecting copper tube with stopcock; (*h*) wire guard around fan blades and centrifuge, the frame clamped to the motor by a metal band (*i*); (*j*) fan; (*k*<sub>1</sub> and *k*<sub>2</sub>) rear and front disks, respectively, of centrifuge. *B*, *C*, outer and inner face views, respectively, of rear centrifuge disk: (*l*) well into which cement flows from copper tube; (*m*) holes through which the cement flows from well in outer face of rear disk into recess of front disk. *D*, inner face view of front centrifuge disk: (*n*) recess in inner face of front disk from which the cement is forced through the lips of the centrifuge.



FIGS. 2-4.—Fig. 2, rubber cement web covering group of emasculated cherry flowers. Fig. 3, moderately heavy web spun over culture of Royal apricot pollen. Note size of pollen grains in relation to size of spaces between the strands of the web. Fig. 4, germination of Royal apricot pollen beneath rubber cement web. Focus of photograph is past the web, hazy outline of which is evident in background.



desirable procedure is to pollinate soon after emasculation, then to spin the web over the branch.

Rubber cements and cement mixtures vary considerably in their ease of spinning, their strength, and their durability under field conditions. The strongest, most durable mixture thus far tested<sup>1</sup> consists of 1 part N-butyl Methacrylate Polymer (dissolved in toluene), 2 parts Goodrich cement No. 1, 2 parts Goodrich cement No. 3, and 4 parts Goodrich cement No. 8540. This mixture is resistant to wind and rain, and has withstood the sun's rays and high temperature for over 10 days. More than sixty rubber cements or cement mixtures have been tested, and improvements in this relation are being sought constantly. Synthetic products have not thus far proved, on the whole, so adaptable to this work as natural rubber.

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<sup>1</sup> I am indebted to The B. F. Goodrich Company for co-operation in working out the many problems relative to the rubber cements.

## CURRENT LITERATURE

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*General Bacteriology*. By D. B. SWINGLE. New York: D. Van Nostrand Company, Inc., 1940. Pp. xii+313. Figs. 157. \$3.00.

This book is designed to give the general science student a well-rounded knowledge of the subject. It assumes that he has had a fundamental course in biology and also has knowledge of elementary chemistry, including organic chemistry.

The subject matter is well balanced and is arranged logically and concisely. The first ten chapters cover the subjects of historical development of bacteriology, microscope and culture methods, morphology, classification, reproduction and growth, nutrition, bacterial products, and effects of the environment on bacteria. Two chapters are devoted to molds, yeasts, and related fungi. The remaining twelve chapters cover the subjects of activities of bacteria in the soil; bacteriology of water supplies, and water examination; the role of bacteria in sewage disposal; microorganisms of the air; milk and milk products; foods; industrial microbiology; infection and immunity.

While the essential points in this broad range of subject matter are well brought out within a minimum of space, a few suggestions might be made. In the discussion of the growth curve of a culture of bacteria (pp. 90-91), the relationship of the *normal* changes in cell size and morphology to the different parts of the curve could have been brought out more clearly. The statements on the composition of the yeast cell wall on pages 156 and 157 appear contradictory. In the chapter dealing with historical development, the subheadings "Discovery of vaccines" and "Discovery of antitoxic sera" might perhaps have been better termed discovery (or development) of the principles of immunization. Chapter XXI on the mechanism of infection seems rather brief. In the chapter on immunity, the discussion of phagocytosis conveys the impression that the white blood cells are the only kind of phagocytic cell. Some explanation of the macrophage and its important role might well have been included.

On the whole the text is relatively free from the minor errors which so often plague first editions. An exception occurs on page 211, however, where the genus *Alcaligenes* is spelled three different ways. The book is well illustrated and many of the illustrations are original.—S. A. KOSER.

*North American Cariceae*. By KENNETH KENT MACKENZIE (posthumous). Illustrated by HARRY CHARLES CREUTZBURG and edited by HAROLD WILLIAM RICKETT. New York: New York Botanical Garden, 1940. Vol. I: pp. iv+1-269, pls. 1-269; Vol. II: pp. 270-547, pls. 270-539.

A sumptuous work, containing descriptions in English, also individual full-page plates, of the 533 species of *Carex* and six other species of Cariceae (three of *Kobresia*, two of *Uncinia*, and one of *Cymophyllus*) previously treated by the late K. K. MACKENZIE in his monograph of the Cariceae (North Amer. Flora 18:1-478. 1931-35). Keys and detailed lists of synonyms are omitted, since these are available in the earlier, monographic treatment. Nor are the descriptions as full as in the earlier work, since many characters have been portrayed by the artist, MR. CREUTZBURG.

A brief note on geographic distribution accompanies each species. This is followed by a precise statement of the one or often several sources, in the herbarium, of material on which the illustration was based—a feature one cannot commend too highly.

This great work may well afford pride to its editor, DR. RICKETT, and to the other authorities of the New York Botanical Garden, to which the author had bequeathed his drawings and a fund to provide for their publication.—E. E. SHERFF.

*Biological Symposia. Vol. I.* Edited by JAKES CATTELL. Lancaster, Pa.: The Jaques Cattell Press, 1940. Pp. vii+238. Illustrated.

The Richmond meeting of the American Association for the Advancement of Science was particularly fortunate in the biological symposia it offered, and this volume presents the papers given at three of these meetings.

Two sessions were devoted to a discussion of the cell theory—the development of the theory and the techniques which have made knowledge of cells possible. The discussion included the modern concepts of the cell as a structural unit, its behavior in development and reproduction, and a philosophical look into the future. The zoologists held a symposium on mating types and their interaction in the ciliate Infusoria. Recent investigations in this line have opened up entirely new fields of research. At a joint session of zoologists and geneticists the subject of chromosome structure was considered. Technical development during recent years and the application of physics and chemistry to the problems have made great advances in the knowledge of this subject possible.

The three biological symposia form a related series on an important theme, and the editor is to be commended for bringing them together in one volume and thus making them available to a larger interested public.—J. M. BEAL.

*Natural History Index-Guide.* Compiled by BRENT ALTSHELER. 2d ed., rev. and enl. New York: H. W. Wilson, 1940. Pp. 583. Sold on service basis.

The difficult problem of quickly locating widely scattered articles in books and periodicals dealing with the field of natural history in its broadest definition is rendered simpler by this index. In all there are 54,063 references to the indexed publications, entered under 9477 subject headings. These headings appear in one alphabetical index with references to material in fifteen major sections: astronomy, atmosphere, botany, food and drink, geography, geology, hygiene and health, microscopy, paleontology, zoology, etc. The compiler was assisted by an able list of collaborators in several scientific institutions.—E. J. KRAUS.

*The Evolution of the Land Plants (Embryophyta).* By DOUGLAS HOUGHTON CAMPBELL. Stanford University, Calif.: Stanford University Press, 1940. Pp. ix+731. Illustrated. \$6.50.

The structure and phylogeny of plants commonly included in the phyla Bryophyta, Pteridophyta, and Spermatophyta are discussed in a general and comparative manner in this large volume.

The classification of the Embryophyta and the range of structure of their gametophytes and sporophytes are first discussed, after which the Bryophyta, Psilophyta, Lycopodiaceae, Equisetaceae, and Filicinae are presented. The Salviniaceae and Marsileaceae are included in one chapter although lack of relationship other than heterospory is acknowledged. Heterospory and the seed habit are ably presented in a separate chapter. Fossil plants receive adequate treatment in the respective plant groups. Less than half the book is devoted to the gymnosperms and angiosperms, with a well balanced treatment of the former. Besides introductory discussions of

the flower and other structures, and of pollination and fertilization in the angiosperms in general and the monocotyledons and dicotyledons in particular, the orders and families of the flowering plants are each discussed briefly. Many families, such as Onagraceae, Oleaceae, and Gentianaceae, are given only a short paragraph each, while the Polemoniaceae and Hydrophyllaceae are grouped together and receive only two sentences of information. As a rule, organographic and anatomical information is given for the orders, while under the family headings only the best known genera are listed and the geographical distribution of the family noted. Genera such as *Phylloglossum*, *Matonia*, and *Dipteris* of the "Pteridophyta" receive a more extensive treatment than in any other single compilation.

The many line drawings are grouped to good advantage, but unfortunately some failed to print clearly or perfectly. At times the research of other authors is mentioned in the text but no citation is given in the bibliography.

The book is to be recommended as a source of general information about the plants under consideration and as a textbook for courses covering many genera without minute detail.—P. D. VORH.

*The New Illustrated Flora of the Hawaiian Islands (Flora Hawaiiensis)*. By OTTO DEGENER. Honolulu, Terr. Hawaii: the author, 1933-1940. Four centuries, unpagged, illustrated, each \$3.50.

This work, of which advance notice was given some years ago in these columns (91:465, 1931), has now been completed through four centuries of plates, with accompanying text. In accordance with the scheme adopted at its inception, its various treatments of both native and introduced plants appear from time to time, on the loose-leaf plan. With the completion of each new century, the subscriber is supplied with an attractive cover and is expected to rearrange all the treatments to date so as to accord with established family sequence. The numerous plates are well executed and mostly from original drawings by artists whom the author has personally trained. Much of the text is based upon the author's own studies or upon recently completed researches of special students of the Hawaiian flora. In this way the author is producing, within the limits of a single work, an increasingly comprehensive and highly authoritative digest of our knowledge of this flora up to the present time.—E. E. SHERFF.

*The Genus Tulipa*. By SIR A. DANIEL HALL. London: The Royal Horticultural Society, 1940. Pp. viii+171. Illustrated.

The opening chapters deal with the history and distribution of the genus, followed by chapters on morphology, cytology, and taxonomy. Since polyploidy is common in *Tulipa*, the cytological studies have cleared up much confusion in nomenclature, have eliminated many synonyms, and assisted toward the establishment of natural subdivisions in the genus.

An interesting feature of the book is that it deals only with species in the living state and which have been studied over a period of years in the collection at the John Innes Horticultural Institution. The author holds strongly that only in this way can the species of a genus like *Tulipa* be properly discriminated. On this account the book is described as a review rather than a monograph, since it embraces critical descriptions of about 100 species only—all that have been in cultivation in western Europe. Other species, almost wholly of central Asiatic origin, which the author was unable to obtain and grow, have been included in an appendix, with a brief summary of their characters derived from their original descriptions.

The book is beautifully illustrated with many colored plates as well as line drawings. It is recommended to all interested in this group of attractive ornamental plants.—J. M. BEAL.

*Flora of Indiana.* By CHARLES C. DEAM. Indianapolis, Indiana: Department of Conservation, Division of Forestry, 1940. Pp. 1236. Illustrated. \$3.50.

This is an exceptionally detailed and critical treatment of a state flora. Distributional ranges are illustrated by 2243 maps. Special features of particular helpfulness to users are the list of more than 700 excluded species; summary of species, varieties, forms, and hybrids; list of new varieties, forms, and combinations published in the text; list of obsolete or not widely used names of localities; reference list of Indiana collectors; list of habitat and distribution terms used; bibliography; and charts showing average dates of last killing frost in spring and first killing frost in autumn for various localities in Indiana.—E. E. SHERFF.

# THE BOTANICAL GAZETTE

December 1940

## USES OF THE FEULGEN REACTION IN CYTOLOGY. II. NEW TECHNIQUES AND SPECIAL APPLICATIONS

B. B. HILLARY

(WITH PLATES I-III)

### Introduction

A previous paper (11) dealt with the effect of fixatives on the Feulgen nuclear stain, the causes of reported negative reactions, and the general applications of the stain. The present paper deals with various new methods made possible by the Feulgen reaction and the use and value of these methods in the study of chromosome form and structure.

The Feulgen reaction has made possible a new and useful method, the squash technique, whereby root tips, ovules, and anthers are fixed and stained *in toto*, and then spread out by squashing into a layer one cell thick. It eliminates the laborious and time-consuming imbedding and sectioning processes. This squash method was first employed by HEITZ (8) with root tips. He called it the *Nucleal-quetschmethode*. The squashing is made possible by the preliminary hydrolysis with HCl, necessary for the Feulgen reaction. This hydrolysis brings about dissolution of the pectic middle lamella and allows the cells to separate.

Squash techniques have numerous special applications and uses. The precise sharp stain, coupled with the spreading and flattening of the chromosomes, has made possible the study of prophase stages in certain plants where they have never been properly seen up to the present. Likewise the results in revealing the spiral structure of the chromosomes have been far superior to those obtained with other stains and methods. In this paper no attempt is made to work out the details of the structure of the chromosomes in any particular form. Rather, examples from various forms are chosen to indicate the wide range of application of the methods.

## Methods

### ANTHER SQUASH

Since the publication of the anther squash technique (9), improvements have been made in the method. The following procedure has been found very satisfactory with material at all stages of meiosis. Anthers are dissected out, fixed with some suitable fixative, and washed. They are stained by means of the Feulgen reaction, bleached in  $\text{SO}_2$  water, and transferred to a drop of 50 per cent acetic acid on a slide. A cover slip is put on and the anthers crushed by tapping and then applying pressure. The preparation is set aside for some time, 10–60 minutes, until it begins to dry at the edges. This period of drying is necessary to allow the loose cells to adhere to the slide and cover slip. Care must be taken not to let the preparations dry out completely, as the material will become desiccated and ruined wherever air comes in contact with it.

After the partial drying the cover slip is lifted off. This can be done with forceps if the cover is left protruding slightly over the edge of the slide. The cell debris is removed and both slide and cover slip dehydrated by flooding with dioxane, which is changed twice at 2-minute intervals. A drop of dioxane balsam is added and the cover slip replaced. If considerable material is present, a new cover slip is put on the slide and the original cover slip put on a new slide in order to ensure that the cells will not overlap.

The value of the anther squash technique lies mainly in the fact that it provides a means of spreading out the chromosomes so that each individual can be seen. This is demonstrated by the photomicrographs (figs. 1–8).

### ANTHER SMEAR SQUASH

With the anther squash method the spiral structure of the chromonemata cannot be studied because pre-treatment before fixation is usually necessary to loosen the chromonematic coils so as to make their structure evident. The pre-treatment methods do not work successfully on whole anthers, so they must be smeared. In smear preparations the chromosomes are usually not spread out and separated sufficiently to allow the best observation. To overcome this the following method is employed. The anthers are smeared on a slide and pre-treated by one of the many methods for this purpose. The most satisfactory results have been obtained with sodium cyanide,  $2^{-5}$  or  $2^{-6}$  molecular solution, applied for 15–30 seconds (a modification of OURA's method, 18). The preparations are then fixed, washed, stained, and bleached. A drop of 50 per cent acetic acid is added to the slide, a cover slip put on, and slight pressure applied. The rest of the procedure is the same as that described for the anther squash technique. The amount of tapping or pressure to be used must be determined by experience because it is easy to apply too much and flatten the chromosomes beyond recognition. Also

there is danger of the chromosomes becoming distorted or damaged if the cover slip is moved laterally owing to tapping with blows not delivered perpendicular to the surface.

#### ROOT TIP SQUASH

The method used by HEITZ with root tips allows only temporary preparations to be made, as they are mounted in acetic acid. Methods of making the preparations permanent, such as those of KLINGSTEDT (15) and DARLINGTON and LA COUR (4), are troublesome and complicated, and in my experience not satisfactory. The details of making permanent root tip squash preparations are given elsewhere (10). This method involves treatment with ammonium hydroxide before hydrolysis, to remove pectic compounds of the cell walls. This additional step is necessary to allow the cells to separate easily when the squashing is carried out in balsam after dehydration with dioxane. Such root tip squash preparations are valuable for making rapid somatic chromosome counts and for study of the details of the morphology of somatic chromosomes.

The root tip squash technique has frequently been criticized because preparations made by this method rarely show polar views of metaphase plates, thus making chromosome counts difficult. This is due to the fact that most of the cells are oriented with their spindles parallel to the long axis of the root. Polar views can sometimes be seen at the growing point, where the cells are not all oriented in the same direction. Frequently polar views can be obtained by moving the cover slip slightly and rolling the cells over. A more certain method is to cut thin freehand transverse sections of the root before fixation. These sections can either be treated by the method given for root tips (10) or they can be squashed in acetic acid, where they can be examined and counts made. If it is desired to make permanent preparations from the acetic acid mounts, the method described for the anther squash works very well. The previous method (10) is preferable for permanent mounts because there is no chance of disarranging or losing any of the cells, as the cover slip is never removed.

The squash method, using transverse sections of the root, is particularly valuable in making chromosome counts in polyploids and plants with large chromosome numbers. The chromosomes are well spread out and they all lie in one optical plane, which is rarely the case with paraffin sections. This is shown by the photograph (fig. 9) of a 60-chromosome *Gladiolus*. BURRELL (2) has recently described a method similar to this but he uses acetocarmine as the stain.

In certain plants the root tips are so small that it is difficult to make freehand sections. With such plants, microtome sections 15–20  $\mu$  thick can be made from imbedded material and mounted on slides in the usual way. After treatment with the Feulgen reaction, they can be squashed in acetic acid and dehydrated in dioxane as before. This allows the chromosomes to be flattened and spread out,



which is impossible in an ordinary section. This method is needed only in extreme cases and usually the ordinary squash method or transverse freehand sections will provide all that is required.

#### OVULE SQUASH

By means of this technique megagametophyte development can be traced from the gynospore mother cell up to fertilization and beyond, without the necessity of cutting sections. It is particularly valuable because the megagametophyte is kept as a unit and not distributed over a number of sections.

The following method has been developed. The ovules are dissected out, fixed, washed, and stained with the Feulgen stain. Vials are used to carry the material through all processes. It is essential to remove as much of the tissue around the ovules as possible. Vascular tissue in particular will carry air and water into the leuco basic fuchsin and oxidize it to basic fuchsin, producing a heavy stain in the cytoplasm which can hardly be removed. The same effect is produced after insufficient washing with a fixative containing formalin. The ovules are transferred from the SO<sub>2</sub> water to a drop of acetic acid on a slide and crushed under a cover slip. Permanent preparations are made in the same way as with the anther squash technique.

It has been possible with this method to trace completely megagametophyte development in *Lilium longiflorum* var. *formosum*. The nuclei and chromosomes stand out vividly in the colorless cytoplasm of the megagametophyte (fig. 10). Mature megagametophytes tend to break and collapse, owing to the large vacuoles present at this stage, but this does not happen in all cases.

How far the application of this technique can be carried is not known. It is limited mainly by the size of the ovules. In many of the angiosperms the ovules were too small to be handled. In *Pinus* the other extreme was found; too much cytoplasm was present, and the egg nucleus—which is not heavily stained owing to its large volume and dispersed chromatin—could not be clearly seen. This technique holds great promise and the limits of its use will be determined only by further investigation.

#### SUMMARY OF METHODS

In summing up the methods given, it may be said that they are all modifications of one general procedure. This is the basic procedure for the squash technique, and in many cases it must be altered or augmented by additional procedures to make it applicable to various types of materials. For the sake of conciseness, this general squash method is listed as follows:

1. Dissect out the structure desired, remove as much of the surrounding tissue as possible, and fix in some suitable fixative.
2. Wash thoroughly (vials are convenient carriers for the material and the fluids

- can be changed with a pipette). If a fixative containing osmic acid is used, bleaching with hydrogen peroxide can be carried out at this stage.
3. Hydrolyze for 5 minutes in normal HCl at 60° C., using a water bath.
  4. Stain in leuco basic fuchsin (fuchsin sulphurous acid) for from 30 minutes to overnight.
  5. Bleach in three changes of SO<sub>2</sub> water of 10 minutes each.
  6. Transfer a single piece of the material to a drop of 50 per cent acetic acid on a slide.
  7. Squash the material under a cover slip by tapping with the end of a pencil and applying pressure with the thumb, using a piece of filter paper to protect the cover slip from finger prints.
  8. Let the preparation stand 10–60 minutes until it begins to dry around the edges of the cover slip.
  9. Remove the cover slip and dehydrate both slide and cover by flooding with dioxane, three changes of 2 minutes each.
  10. Add a drop of Canada balsam and replace the cover slip.

#### Special applications

TO REVEAL SATELLITES.—The demonstration of satellites is a matter of proper fixation rather than of the technical methods employed. With certain fixatives they show up well in sectioned or smeared material, but frequently they are lying under another chromosome, or some similar condition obscures them. This difficulty can usually be overcome by means of the squash methods. With root tip squashes the chromosomes of the complement can be so well spread out that each individual is separate from the others. The same applies to pollen grain smears at the time of the first mitosis.

The smear squash method has proved very satisfactory with pollen grains, since they do not become contracted and shriveled as is frequently the case when balsam is added. This is probably due to the fact that a large area of the pollen grain wall is stuck to the glass, thus holding it there and allowing the balsam time to penetrate and fill the large vacuole before collapse of the wall can take place.

In mitotic prophase preparations stained with the Feulgen method the nucleolus is colorless, in contrast to the dense color it takes with other stains. This enables the satellites to stand out clearly against the colorless body of the nucleolus, showing their structural details. Yet it is often convenient to have some means of locating the satellites. This can be done by means of a counter stain of fast green, which stains the nucleolus a pale transparent green and does not interfere with the Feulgen stain of the chromosomes (fig. 11). The counter stain can be applied by placing the slide or material, following the SO<sub>2</sub> bleach and washing, in a 0.1 per cent solution of fast green for from 2 hours to overnight. They are then rapidly

washed, and the squashing in acetic acid and subsequent treatment carried out. SEMMENS and BHADURI (19) describe a technique for the differential staining of nucleoli and chromosomes by means of the Feulgen reaction and light green. Their method seems unnecessarily complicated, and in my experience the results obtained are no better than those with the simple aqueous solution. Fast green is used rather than light green because it is less likely to fade.

Normally with the Feulgen stain I use color filters to increase the contrast and also the definition. The combination of Ilford 3 and 5 or Wratten B and E filters (green and orange) has been found to give the best results. When these combinations are used with nucleoli stained with fast green, however, they are almost invisible because of low contrast. The orange filter alone gives a satisfactory contrast in chromosomes, satellites, and nucleoli. Other combinations also give excellent contrast (figs. 11, 12).

TO CLEAR UP MEIOTIC PROPHASE STAGES.—The anther squash method is particularly valuable for study of the prophase stages of meiosis. This method is indispensable in many plants, such as certain Liliaceae, where it is difficult to smear the anthers in early prophase since the cells tend to stick together in a tissue formation and will not adhere to the slide. Nuclei at the early prophase stages can be flattened and the chromonematic threads well spread out. They are not pulled out of place or distorted as is frequently the case with smears. The selective staining of the Feulgen reaction clearly reveals chromomeres of different sizes and shapes, with an unstained portion of the chromosome thread between them (fig. 13). KAUFMANN (14), KOSHY (16), and NAITHANI (17) attribute the beaded appearance of the chromosome to the intertwining of two threads, the chromomeres representing merely optical twists of the threads which have been exaggerated by the action of the fixatives. If such were the case the portion of the thread between the chromomeres would be stained. The Feulgen reaction demonstrates the presence of chromomeres and shows that they have a different chemical composition from that of the rest of the chromosome thread.

This technique also shows clearly the details of the chiasmata at diplotene. With especially favorable material, like the spermatocytes of grasshoppers, the crossing-over of the chromonemata is so clear that it appears almost diagrammatic (fig. 14). On the other hand, in some forms such as *Gasteria* it has been impossible up to the present to obtain a clear picture of chiasmata, owing to the fact that the chromosomes are labile and tend to run together, losing their individuality. In such forms the diplotene and diakinesis stages are usually referred to as the diffuse stage. With the Feulgen stain, used after Carnoy's fixative, which fixes the chromosomes before they get a chance to coalesce, it is possible to discern the chiasmata. A comparison of figure 15 with the drawings of TAYLOR (20) and TUAN (22) of a similar stage in *Gasteria* will make this clear. This method may prove useful in

forms such as *Tradescantia* and *Oenothera*, where preparations of the diplotene stage have been unsatisfactory. Preliminary work has been carried out on these forms with promising results.

TO STUDY CHROMOSOME STRUCTURE.—Preparations showing the spiral structure of the chromosomes can be produced by the pollen mother cell smear method and particularly the smear squash method. With the Feulgen stain the chromonema is revealed clear cut and sharp in a colorless matrix. This finding is opposed to most, if not all, previous reports. HUSKINS and SMITH (12) and GEITLER (6) report that the Feulgen nucleal stain gives no differentiation between the chromonema and its matrix. BAUER (1) and ZEIGER (24) report that both matrix and chromonema are nucleal positive. In no case in which material was fixed to reveal the spiral structure of the chromosomes was the matrix stained. It might be argued that in a chromosome which does not show a coiled chromonema, the matrix is also stained, since the whole chromosome appears homogeneously stained. The homogeneous appearance is almost certainly due to the tight coiling of the chromonema, which prevents the resolution of the different gyres. When the matrix is swollen as the result of pre-treatment, the coils are loosened and the gyres can be resolved; then the chromonema can be distinguished from the matrix. In cases where fixation has resulted in a vacuolated structure, this is of course revealed by the stain. Such a structure must be looked upon as an artifact.

With such Feulgen stained material the details of both major and minor coils at the first metaphase and anaphase are revealed (figs. 16, 17). The separation of the daughter chromonemata at the onset of the anaphase shows up sharply. Coiling in second meta- and anaphase chromosomes (that is, the minor coil), which has been revealed in comparatively few cases and then only by the aceto-carmine method of temporary staining, can be shown with the Feulgen stain (fig. 18).

The question of coiling in somatic chromosomes has led to marked divergence of opinion. The view that the chromonemata form two interlocking coils in the anaphase has been advanced by KAUFMANN (13), KOSHY (16), NAITANI (17), TELEZYNSKI (21), TAYLOR (20), TUAN (22), and others. GEITLER (5, 7), UPCOTT (23), and DARLINGTON and LACOUR (4) have reported and illustrated a single coiled structure essentially similar to that found in meiosis. The aceto-carmine method was used by GEITLER and UPCOTT to reveal this structure, while DARLINGTON and LACOUR used the Feulgen method. Judging from the photographs of the latter, the results obtained have not been particularly favorable. In this laboratory the single coiled structure has been demonstrated through the use of the Feulgen method by COLEMAN (unpublished) in the pollen mitosis of *Gasteria trigona*, and by GOPAL-AYENGAR (unpublished) in the pollen tube mitosis of *Scilla* species.

Other stains will stain the chromonemata of chromosomes, but they are not so selective, and the preparations lack the precise staining so characteristic of the Feulgen stain and so necessary for a study of the details of chromosome structure. Moreover, by means of the Feulgen stain the chemical differentiation of the chromonema from its matrix has been definitely demonstrated.

WITH TRYPTIC DIGESTION.—Recent investigations by CASPERSSON (3) have shown that it is possible by means of tryptic digestion to remove the nucleoproteids from the chromosome, leaving only the thymonucleic acid. The digestion also removes any extra-chromosomal proteids in the nucleus as well as those of the cytoplasm. CASPERSSON found, by treating cells with lanthanum acetate, that the nucleic acid could be precipitated as an insoluble compound which he calls lanthanum nucleinate. The material is then subjected to digestion by the enzyme trypsin, which removes the protein of the matrix but leaves the thymonucleic acid intact.

CASPERSSON studied mainly the salivary gland chromosomes of *Drosophila* and the spermatocytes of certain grasshoppers. He did this by means of photomicrographs in ultra-violet light with wave lengths of 2560–2750 Å. The differentially greater absorption of light rays of this wave length by nucleic acid led to photomicrographs showing great detail in the bands of salivary gland chromosomes, to which areas the thymonucleic acid is restricted. Previous digestion gave preparations which under the ultra-violet microscope resulted in great detail and clearness. CASPERSSON did not use his technique in a study of chromosome structure in plant cells.

As ultra-violet microscope equipment is expensive and therefore not generally available, it was felt that the possibility of combining CASPERSSON's digestion method with the Feulgen technique should be investigated. It had first to be ascertained whether the precipitation of thymonucleic acid as a lanthanum salt interferes with the action of hydrolysis. Experiments showed that this is not the case and that the Feulgen reaction can be carried out successfully on material treated by CASPERSSON's digestion method.

A modification of CASPERSSON's method was introduced. Instead of using a mixture of fresh trypsin from the mucous lining of pig's gut and enterokinase from pig pancreas, a dried commercial preparation of trypsin was found to be equally satisfactory. Smears of pollen mother cells of *Tradescantia virginiana* were pre-treated and fixed as before. The slides were then left overnight in a 0.1 per cent solution of lanthanum acetate. They were next placed in a 1 per cent solution of trypsin which contained a trace of lanthanum acetate, to prevent dissolution of the thymonucleic acid. They remained in this solution for 24 hours at 37° C. The trace of lanthanum acetate necessary for the reaction was provided by not washing the slides when transferring from the lanthanum acetate solution to the trypsin

solution. When five slides were placed in a Coplin jar at one time, sufficient lanthanum acetate was carried along to keep the nucleic acid in an insoluble form.

This combination of time of digestion and concentration of trypsin was sufficient to remove most of the matrix of the chromosomes and some of the cytoplasm. By increasing the time or concentration more complete digestion could be brought about, but if it was carried too far there was nothing left to hold the chromonemata to the slide. Preparations treated in this way were a considerable improvement over non-digested preparations. The clear but light refracting cytoplasm of the background is removed and the halo effect of the matrix is gone, leaving the chromonemata standing out naked and sharp. There is nothing to obscure the most minute details of the structure and coiling of the chromonemata; thus this type of preparation is unequalled for investigation of chromosome structure. For ordinary work such a highly refined smear technique is not necessary, but for future investigation of chromosome structure this method should prove valuable.

### Summary

1. Procedures are described for use with the squash technique, whereby anthers, ovules, and root tips are fixed and stained *in toto*, then spread out into a layer one cell thick by squashing.
2. The smear squash method of studying chromosome structure, particularly the minor coil, is described. Contrary to most previous reports, the Feulgen stain was found to stain only the chromonema and not the matrix of the chromosome.
3. The Feulgen stain can be used with marked success after the trypsin-lanthanum digestive method of CASPERSSON, which removes the matrix of the chromosomes as well as other cell proteids. Preparations made in this way are particularly sharp and clear, and this method holds promise for the investigation of chromosome structure.
4. The anther squash technique is the first to reveal at all clearly the chiasmata and individuality of the chromosomes in forms that have a diffuse stage at diplotene and diakinesis.
5. Satellites can be revealed with remarkable clarity by the root tip squash method, and the anther smear squash method when used with pollen grains. A counter stain of fast green is used to differentiate the nucleolus.

The writer wishes to express thanks to Dr. L. C. COLEMAN for assistance and the use of many of his preparations and to Dr. D. H. HAMLY for help with photography.

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## EXPLANATION OF PLATES I-III

## PLATE I

FIGS. 1-6.—Anther squash preparations of *Lilium longiflorum* var. *formosum* fixed in Carnoy's fluid and hydrolyzed 5 minutes. All  $\times 1000$ .

FIG. 1.—Pachytene stage.

FIG. 2.—Early diplotene showing crossing-over of chromonemata.

FIG. 3.—Diakinesis; chromosomes well spread out.

FIG. 4.—Meta-anaphase.

FIG. 5.—Anaphase I showing chromosomes well spread out.

FIG. 6.—Interphase showing opening out of major and minor coils.

## PLATE II

FIG. 7.—Metaphase II of *L. longiflorum* var. *formosum* showing complete chromosome complement with chromosomes (some terminal and others median) attached at primary constriction.  $\times 1000$ .

FIG. 8.—Anaphase II of same, with chromosomes well spread out.  $\times 1000$ .

FIG. 9.—Root tip squash preparation of 60-chromosome *Gladiolus*, fixed in Carnoy. (From preparation made by G. S. SWAIN.)  $\times 1500$ .

FIG. 10.—Ovule squash preparation of *Lilium longiflorum* var. *formosum* showing megagametophyte with 12- and 36-chromosome nuclei at metaphase. Belling's fixative.  $\times 500$ .

FIG. 11.—Pollen grain smear of *Aloe vera* showing satellites on nucleolus. To bring out sufficient contrast between the chromosomes and nucleolus, which is stained with fast green, the following color filters were used: Ilford 5, Wratten E, and Chant's signal green glass. Fleming-Heitz fixative.  $\times 1400$ .

FIG. 12.—Same nucleus as in fig. 11 taken with ordinary color filter combination used with Feulgen stain (Ilford 3 and 5). Nucleolus scarcely visible.

FIG. 13.—Anther squash preparation of *Veltheimia viridifolia* showing zygotene stage. Chromomeres appear as beads with unstained portion of thread between them. Fixed in Carnoy.  $\times 1500$ .

## PLATE III

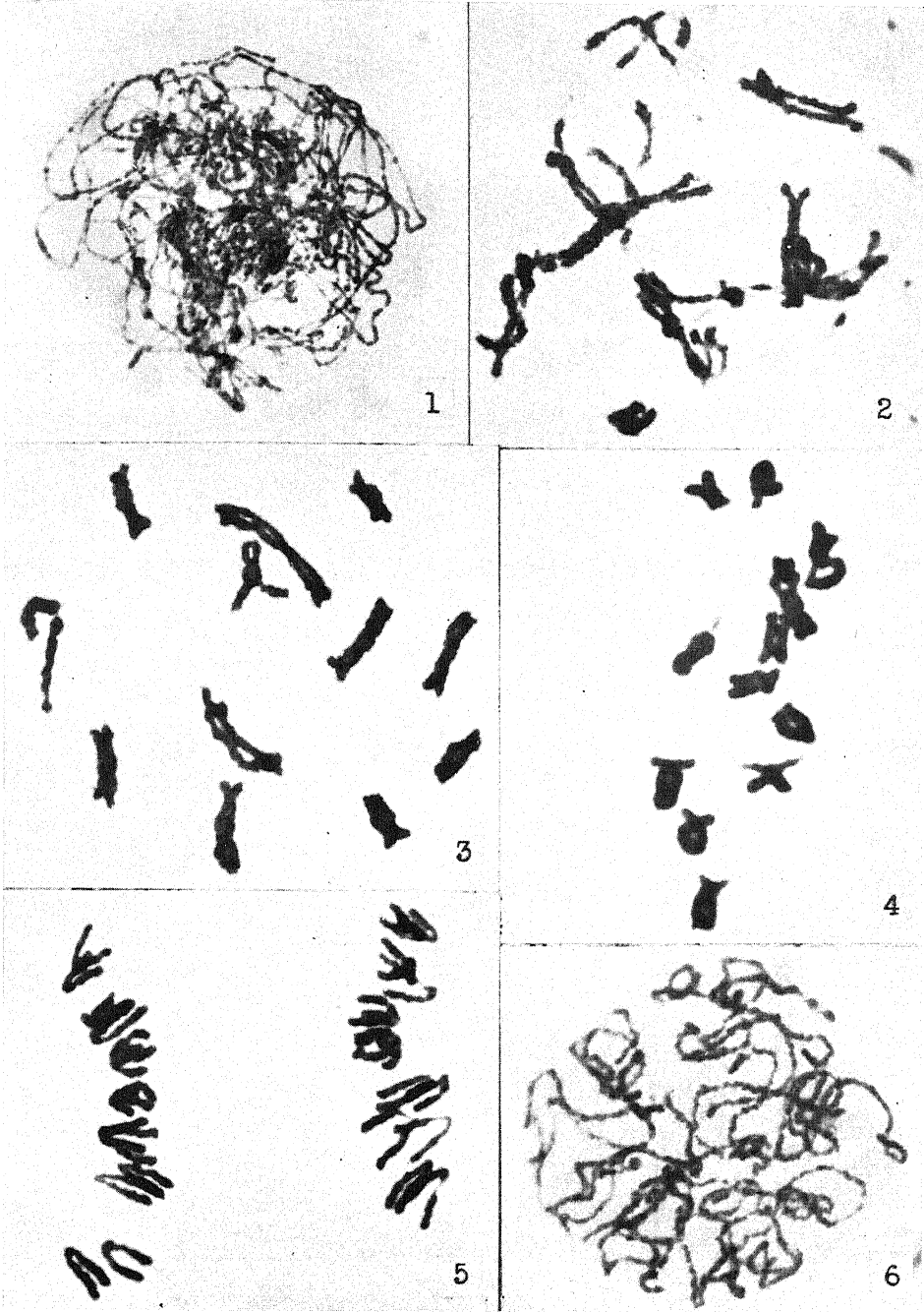
FIG. 14.—Serial photographs of different focal levels of diplotene stage of the grasshopper, *Melanoplus femur rubrum*, showing well defined chiasmata. Preparation made by squashing the testes tubules in the same way as anthers.  $\times 1000$ .

FIG. 15.—Anther smear squash of the rarely seen diplotene stage of *Gasteria trigona*, showing chiasmata and chromosomes well spread out. Fixed in Carnoy.  $\times 1000$ .

FIG. 16.—Anther smear squash of *Tradescantia virginiana* at metaphase I, showing minor coils of double chromonemata and connections between chromosomes in ring of four. Pre-treated 30 seconds with NaCN 2<sup>-s</sup> molecular solution and fixed in Carnoy.  $\times 1600$ .

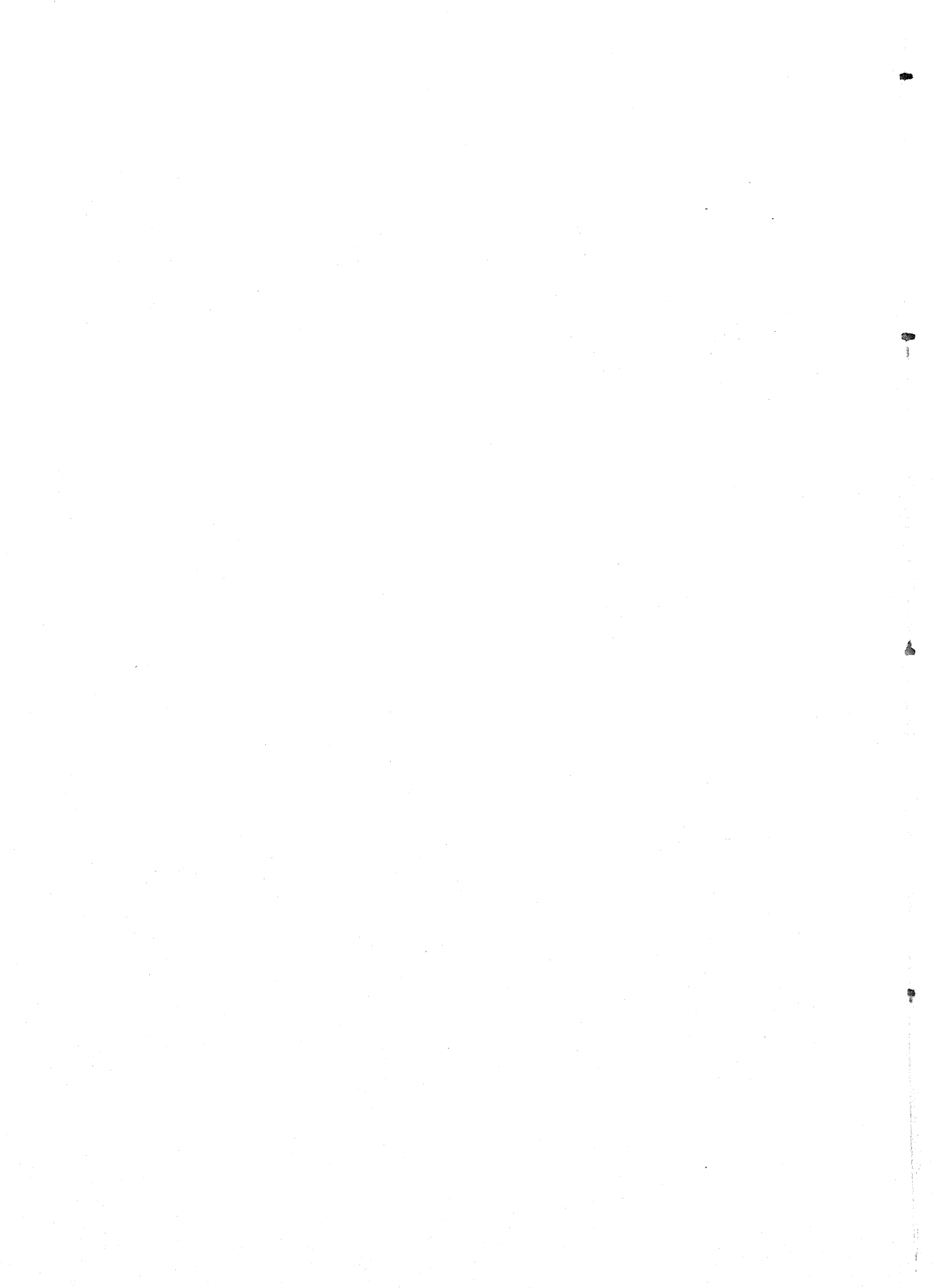
FIG. 17.—Anther smear squash of early anaphase I of *T. paludosa* with minor coil revealed at points indicated by arrows.  $\times 1300$ .

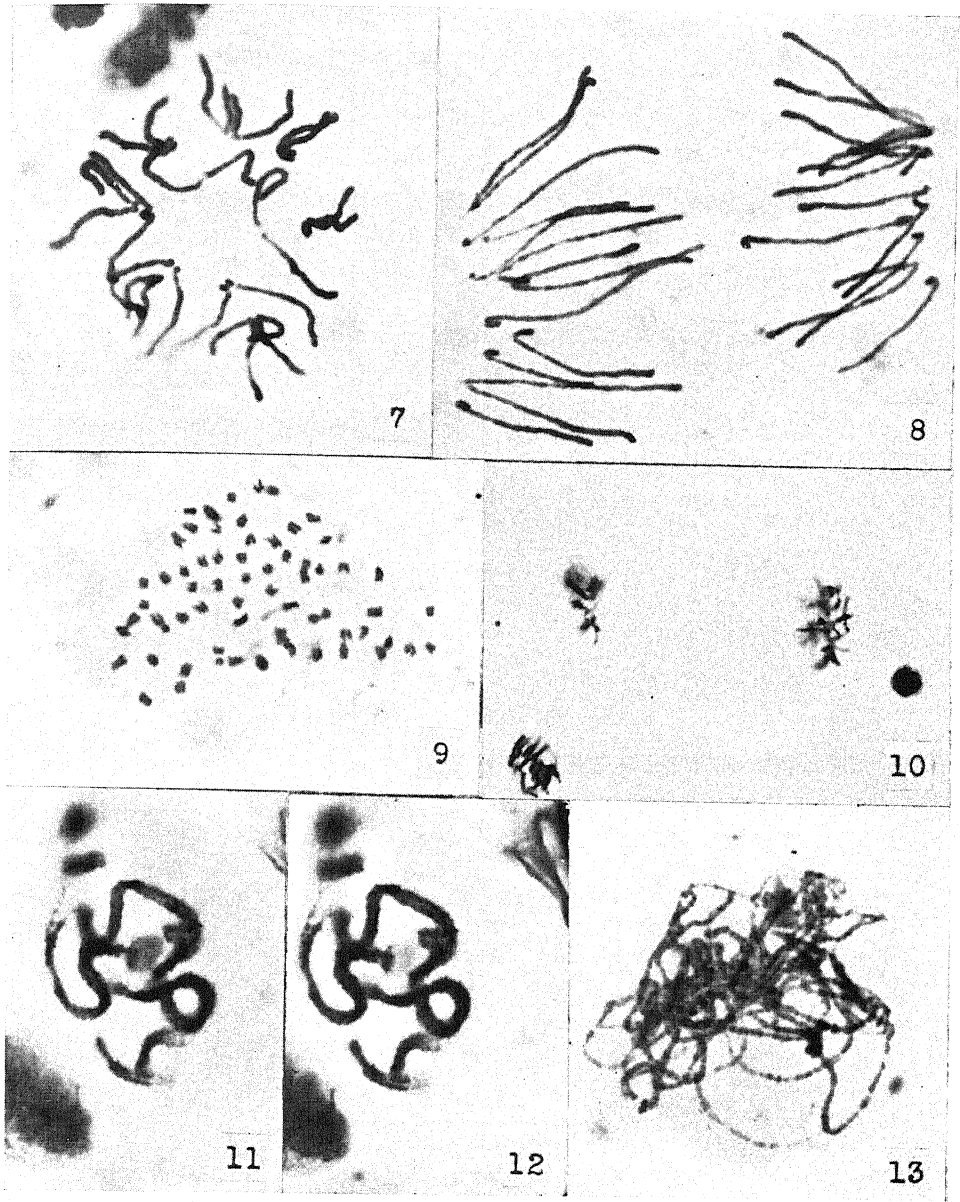
FIG. 18.—Anther smear squash of anaphase II of same, showing minor coil. Pre-treated in NaCN 2<sup>-s</sup> molecular solution for 15 seconds and fixed in Navashin.  $\times 1500$ .



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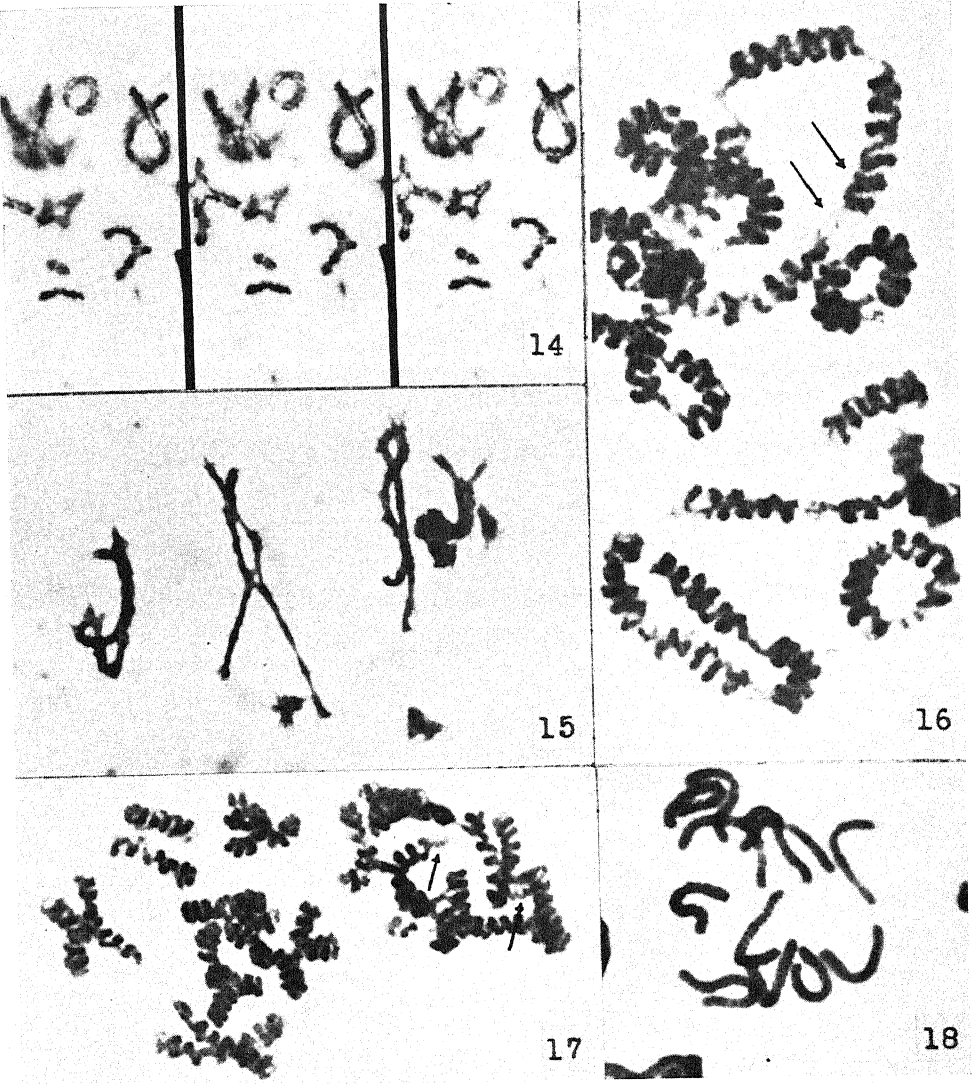






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# STUDIES OF CHROMOSOMAL ASSOCIATION AND BEHAVIOR AND OCCURRENCE OF ANEUPLOIDY IN AUTOTETRAPLOID GRASS SPECIES, ORCHARD GRASS, TALL OAT GRASS, AND CRESTED WHEATGRASS<sup>1</sup>

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(WITH THREE FIGURES)

## Introduction

Although most polyploid species behave cytologically as allopolyploids, some appear to be autopolyploids. In an extensive review of the literature on this subject, MÜNTZING (8) emphasized the importance of autopolyploidy in evolution.

Among the many polyploid species of the Gramineae, at least four have been reported to show meiotic chromosome behavior characteristic of autopolyploids. The presence of quadrivalents at metaphase I has been reported by MÜNTZING (7, 9) in orchard grass (*Dactylis glomerata*), by VON BERG (1) in *Hordeum bulbosum*, and by KATTERMANN (3) in tall oat grass (*Arrhenatherum elatius*) and sweet vernal grass (*Anthoxanthum odoratum*). PETO (12) found both diploid and tetraploid races in crested wheatgrass (*Agropyron cristatum*), and the senior writer (unpublished) observed the regular occurrence of quadrivalents at meiosis in the tetraploid race of this species.

Cytological studies of naturally occurring autopolyploids are of both theoretical and practical importance. A comparison of the meiotic behavior of different species and of plants within these species should throw light on the effects of natural selection on chromosomal behavior. Such information will be particularly valuable for comparisons with induced autopolyploids. From a practical standpoint, a varietal improvement program can be outlined more intelligently when the cytogenetical behavior of the species is understood.

## Material and methods

Microsporocytes were collected from six plants of crested wheatgrass, ten plants of tall oat grass, and three plants of orchard grass. The sporocytes were

<sup>1</sup> The quadrivalent frequency and percentage of metaphase I sporocytes showing univalents were determined for crested wheatgrass plants GH-1 and GH-2 and tall oat grass plants 162-1, 162-3, 162-4, 162-6, 162-10, and 162-11, while the senior writer was a member of the staff of the Division of Agronomy and Plant Genetics, University of Minnesota. All other phases of the investigation were conducted at the U.S. Regional Pasture Research Laboratory, State College, Pennsylvania.

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fixed in acetic-alcohol (three parts absolute alcohol to one part glacial acetic acid) and left in this solution until examined. Aceto-carminic smear slides were used, and all data were recorded and photomicrographs made from the fresh slides. Best preparations were obtained from material killed the previous day, but fairly satisfactory slides were made from material that had been in the fixing solution more than a month.

The chromosome numbers of 116 plants of orchard grass were determined from paraffin sections of root tips, prepared as described previously for *Lolium perenne* (10). In determining the number of chromosomes, three or more of the best plates from each plant were sketched and the counts made from these sketches. In case of discrepancy the plates were reexamined or new ones sketched until the count seemed correct. In the tall oat grass and crested wheatgrass plants which were studied, chromosome numbers were determined from the microsporocytes.

### Experimental results

#### CHROMOSOME NUMBERS

Of the 116 plants of orchard grass studied, 59 per cent had the euploid chromosome number of 28. The remaining 41 per cent were aneuploid, 22 per cent having 27, 12 per cent 29, and 7 per cent 30 chromosomes. Of the ten plants of tall oat grass studied, nine were euploid ( $2n = 28$ ) and one had 27 chromosomes. Five of the crested wheatgrass plants had 28 chromosomes while one showed clearly more than 28. In this plant the chromosome number at diakinesis could be determined unquestionably in only two sporocytes, both of which showed 31 chromosomes.

#### MEIOTIC BEHAVIOR

MID-PROPHASE.—The chromosomes at mid-prophase were found to be regularly associated in pairs but frequent changes of partners were observed, indicating some quadrivalent associations. Although it was not possible to analyze a complete nucleus at this stage, in some cases a single pair of homologues could be seen associated throughout their entire length, a type of pairing which should result in a bivalent at diakinesis and metaphase I. Quantitative data on the frequency of such pairing could not be obtained, but it is doubtful whether it occurs frequently enough to account for all the bivalents produced. Bivalents probably arise also as a result of failure of chiasma formation in certain paired segments of quadrivalent associations in prophase.

DIAKINESIS AND METAPHASE I.—The frequency of quadrivalents and bivalents was determined from diakinesis, metaphase I, or both. When both stages were used in a particular plant, the results were similar. The data were therefore combined. Only nuclei which could be analyzed completely were recorded. The num-



ber of sporocytes in each plant showing various combinations of bivalents and quadrivalents, the average number of quadrivalents per plant, and the total and average for each species are given in table 1. All possible combinations of bivalents and quadrivalents, except 14 bivalents, were observed. In addition to

TABLE 1  
NUMBER OF MICROSPOROCTES SHOWING VARIOUS NUMBERS OF  
BIVALENTS AND QUADRIVALENTS

PLANT NO.	$1^2II+1^1IV$	$1^0II+2^1IV$	$8II+3IV$	$6II+4IV$	$4II+5IV$	$2II+6IV$	7IV	TOTAL NO. OF CELLS	AVERAGE NO. OF IV
ORCHARD GRASS									
11(13).....	1	5	4	11	6	2	0	29	3.8
22(2).....	3	2	2	11	6	4	2	30	4.2
138(3).....	1	1	4	4	1	0	0	11	3.3
Total.....	5	8	10	26	13	6	2	70	3.9
CRESTED WHEATGRASS									
GH1.....	1	4	6	6	4	1	1	23	3.7
GH2.....	2	6	5	5	6	1	0	25	3.4
4(1).....	0	0	0	1	0	0	0	1	.....
4(4).....	0	1	1	0	0	0	0	2	.....
9(1).....	1	1	3	5	5	3	0	18	4.2
Total.....	4	12	15	17	15	5	1	69	3.7
TALL OAT GRASS									
162-1.....	1	1	7	12	5	1	0	27	3.8
162-3.....	0	2	5	4	2	0	0	13	3.5
162-4.....	0	3	4	5	5	0	0	17	3.7
162-6.....	0	4	12	10	3	0	0	29	3.4
162-11.....	1	4	8	9	8	2	3	35	4.1
9(7).....	1	0	0	3	3	1	0	8	4.2
12(3).....	0	2	1	3	4	2	1	13	4.5
12(1).....	0	0	1	1	1	1	0	4	4.5
4(4).....	1	3	10	6	8	1	1	30	3.8
Total.....	4	19	48	53	39	8	5	176	3.8

the 315 sporocytes recorded in table 1, hundreds of nuclei at diakinesis and metaphase I were examined in which complete analysis was not possible. Since all these nuclei had one or more quadrivalents, sporocytes with 14 bivalents must occur rarely if at all. Sporocytes in diakinesis in orchard grass are shown in figure 1A, B. The quadrivalents and bivalents are clearly visible. The occurrence of 7

quadrivalents in some nuclei of each species indicates at least partial homology within each of the 7 sets of 4 in each species. In tall oat grass 162-11, of 76 nuclei examined at diakinesis one had a ring of 12 chromosomes plus 4 quadrivalents. This was the only sporocyte in any plant in which an association of more than 4 chromosomes was observed, indicating that the ring of 12 was the result of an aberration which occurred in a premeiotic division or in prophase of meiosis. The production of such a ring in this material would require two interchanges with one set of homologues in common.

Although the mean numbers of quadrivalents for the three species were very nearly alike, variation between plants within each species was found. The range of means for plants of orchard grass was 3.3 to 4.2, for crested wheatgrass 3.4 to 4.2, and for tall oat grass 3.4 to 4.5. Since determinations for a plant were made

TABLE 2  
PERCENTAGE RING AND CHAIN QUADRIVALENTS SHOWING ADJACENT  
AND ALTERNATE TYPES OF ORIENTATION AT METAPHASE I

TYPE OF ORIENTATION	TALL OAT GRASS (PLANT NO.)					CRESTED WHEATGRASS (PLANT NO.)		
	162-1	162-4	162-6	162-11	TOTAL	G.H. 1	G.H. 2	TOTAL
Alternate.....	71.4	70.0	61.8	90.7	74.4	79.1	90.6	84.7
Adjacent.....	28.6	30.0	38.2	9.3	25.6	20.9	9.4	15.3
Number of quadrivalents.	77	66	68	86	297	91	85	176

usually from a single collection of sporocytes and since the sporocytes were collected at different times, no valid estimate of error is available. It is impossible, therefore, to evaluate the significance of these differences between plants. Nevertheless they are suggestive of a possible trend in natural selection for different quadrivalent frequencies.

The majority of the quadrivalents observed at diakinesis and metaphase I were simple rings or chains with completely or nearly completely terminalized chiasmata. The type of orientation of these rings and chains in four plants of tall oat grass and two plants of crested wheatgrass was classified as to whether alternate or adjacent chromosomes were directed toward the same pole (table 2). Figure 1C shows a quadrivalent of the adjacent type and figure 1D shows quadrivalents the members of which are oriented in an alternate manner. The four plants of tall oat grass ranged from 61.8 to 90.7 per cent of the quadrivalents oriented alternately, while in the two plants of crested wheatgrass, 79.1 and 90.6 per cent of the quadrivalents were classed as alternate. The magnitude of the differences suggests that they may be significant.

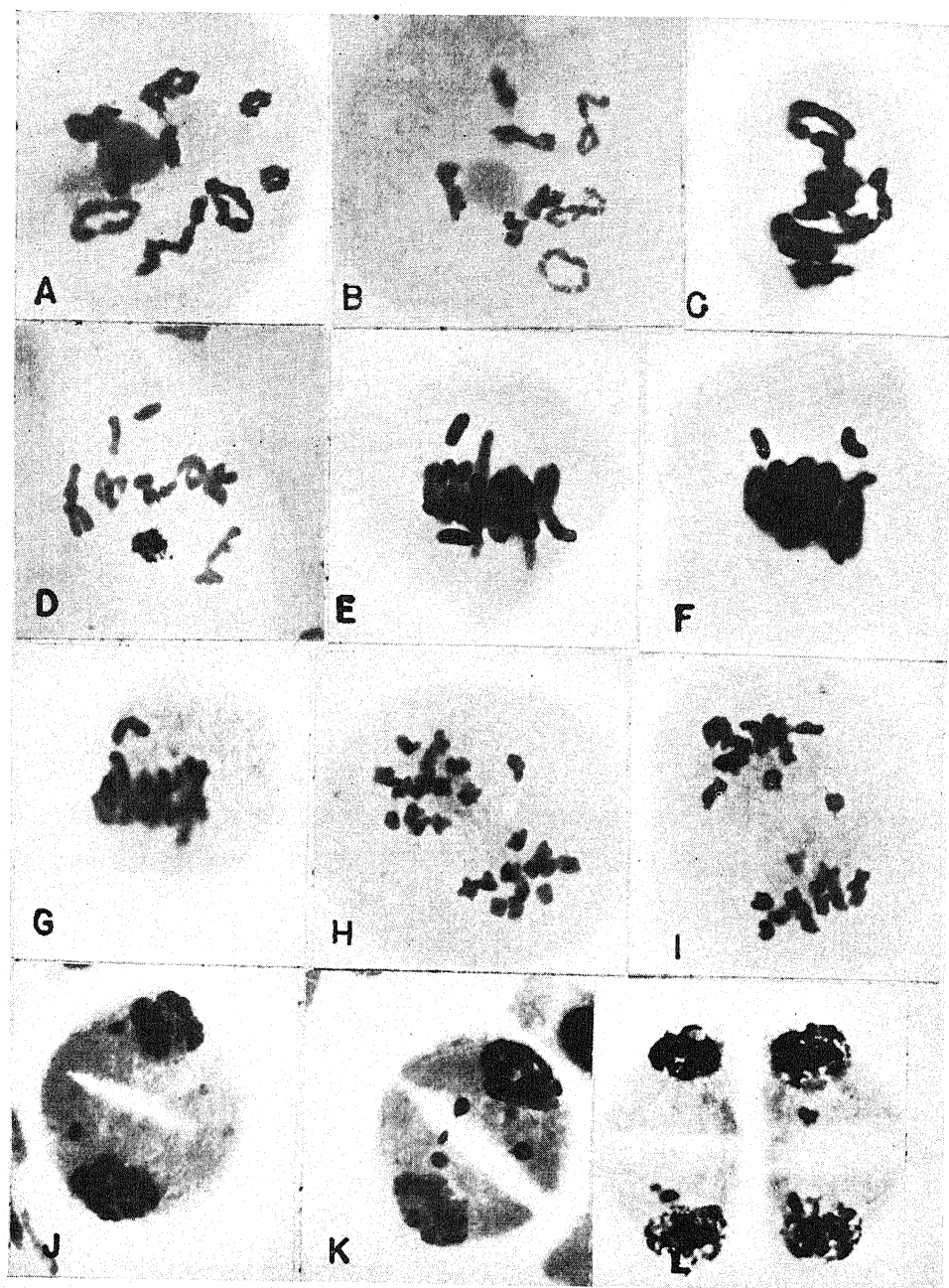


FIG. 1.—*A, B*, quadrivalents and bivalents at diakinesis; *C, D*, types of quadrivalents at metaphase I; *E, F, G*, univalents at metaphase I; *H, I*, lagging and dividing univalents at anaphase I; *J, K*, micronuclei at interphase I; *L*, quartet showing planes of meiotic divisions.

In addition to the sporocytes having only bivalents and quadrivalents, some were observed with one or more univalents at metaphase I. In obtaining quantitative data on percentage of metaphase I sporocytes with different numbers of univalents (table 3), an attempt was made to record only univalents which apparently had not been associated with other chromosomes by chiasmata. Some errors undoubtedly were made in distinguishing these univalents from those resulting from precocious disjunction. Also, univalents probably were obscured by the main group of chromosomes in some nuclei, so that the percentages given in table 3 may represent minimum values for that particular group of sporocytes. Large differences in the univalent frequency in different plants were found. Tall oat grass 12(3) was the only plant in the three species in which univalents were not observed, although the percentage of sporocytes with univalents was low in plants 162-3 and 162-11. The greatest frequency of single univalents was found in crested wheatgrass 4(6), but the percentage of nuclei showing one or more univalents was higher in 4(1). The frequency of metaphase I plates showing two univalents is considerably higher than would be expected owing to chance—on the basis of the frequency of nuclei with single univalents. This may have resulted from conditions within a given nucleus tending to decrease chiasma formation. Thus such a nucleus would frequently have two or more univalents which would not necessarily be homologous. In sporocytes with two univalents, however, the univalents were usually similar morphologically (fig. 1E, F), whereas those seen in different nuclei were frequently strikingly different in size and arm length (fig. 1G). Attempts to identify the chromosomes at this stage are admittedly unsafe. Nevertheless these observations suggest that the two univalents in a particular nucleus were frequently homologous. This indicates that when pairing in a homologous set at prophase is such that one chromosome fails to form a chiasma, there is a tendency for another chromosome of the same set to be excluded from chiasma formation, with the resulting formation of two univalents and one bivalent instead of one univalent and one trivalent.

ANAPHASE I.—In all plants in which anaphase I figures were examined, some showed one or more lagging univalents (fig. 1H, I). All univalents which seemed to be lagging sufficiently to prevent their inclusion in one of the daughter nuclei were on the equatorial plate or close to it and were clearly split longitudinally. In many cases the half chromosomes had already disjoined and were seen lying close to the two daughter chromosome groups. As may be seen in table 3, there is a tendency for the frequency of lagging univalents at anaphase I to be higher in those plants with a high frequency of univalents at metaphase I, indicating that unpaired chromosomes are unable to move in a normal manner to the pole, but lag behind, split equationally, and move to the poles as half chromosomes. It is impossible to estimate from these data what proportion of

the metaphase I univalents behave in this manner. Tall oat grass 12(3), which did not have univalents in any of the 184 metaphase I figures counted, had one lagging univalent in 5.2 per cent of the anaphase I nuclei examined. All plants except tall oat grass 12(1) showed a corresponding increase in frequency of ana-

TABLE 3

PERCENTAGE OF METAPHASE I SHOWING DIFFERENT NUMBERS OF UNIVALENTS AND PERCENTAGE OF ANAPHASE I SHOWING DIFFERENT NUMBERS OF LAGGING UNIVALENTS

PLANT NO.	METAPHASE I							ANAPHASE I							
	No. EX- AMINED	No. OF UNIVALENTS						No. EX- AMINED	No. OF LAGGING UNIVALENTS						
		1	2	3	4	6	TOTAL		1	2	3	4	6	8	TOTAL
ORCHARD GRASS															
11(13).....	102	6.9	3.0	.....	.....	.....	9.9	55	9.1	9.1	.....	.....	.....	.....	18.2
22(2).....	172	5.8	0.6	.....	.....	.....	6.4	48	14.6	8.3	2.1	.....	.....	.....	25.0
138(3).....	119	16.8	9.2	2.5	0.8	1.7	31.0	16	18.8	12.5	6.2	6.2	6.2	6.2	56.1
CRESTED WHEATGRASS															
GH 1.....	500	7.0	0.4	.....	.....	.....	7.4	.....	.....	.....	.....	.....	.....	.....	.....
GH 2.....	356	2.5	0.6	.....	.....	.....	3.1	.....	.....	.....	.....	.....	.....	.....	.....
4(1).....	103	16.5	16.5	2.0	4.0	.....	39.0	84	30.9	3.6	1.2	.....	.....	.....	35.7
4(4).....	121	8.2	10.7	3.3	0.8	.....	23.0	60	16.7	3.3	.....	.....	.....	.....	20.0
4(6).....	142	22.5	4.2	5.6	.....	.....	32.3	11	36.4	9.1	.....	.....	.....	.....	45.5
9(1).....	135	18.5	11.1	.....	.....	.....	29.6	3	66.0	.....	.....	.....	.....	.....	66.0
TALL OAT GRASS															
162-3.....	151	0.7	.....	.....	.....	.....	0.7	.....	.....	.....	.....	.....	.....	.....	.....
162-10.....	661	7.3	2.0	.....	.....	.....	9.3	.....	.....	.....	.....	.....	.....	.....	.....
162-11.....	600	0.8	0.5	.....	.....	.....	1.3	.....	.....	.....	.....	.....	.....	.....	.....
4(4).....	168	3.0	3.0	.....	.....	.....	6.0	88	8.0	9.1	1.1	.....	.....	.....	18.2
9(7).....	111	4.5	1.9	.....	.....	.....	6.4	60	11.7	6.7	3.3	.....	.....	.....	21.7
12(1).....	151	10.6	2.0	.....	.....	.....	12.6	168	3.0	.....	.....	.....	.....	.....	3.0
12(3).....	184	.....	.....	.....	.....	.....	0.0	135	5.2	.....	.....	.....	.....	.....	5.2

phase I with single laggards as compared with the percentage of single univalents at metaphase I. When the total percentage of anaphase I nuclei showing one or more lagging univalents is compared with the total percentage of metaphase I with one or more univalents, a similar increase is noted in all plants except tall oat grass 12(1) and crested wheatgrass 4(1) and 4(4). In the latter two plants the differences between anaphase I and metaphase I were slight and may not be

significant. It seems probable that in some of the plants studied, lagging univalents arose from some source other than unpaired chromosomes at metaphase I. It is possible that in quadrivalents, particularly those the members of which are oriented in an adjacent manner, the disjoining chromosomes lie so far apart that forces of repulsion will be insufficient to move all chromosomes to the poles. Such chromosomes would lag and perhaps undergo longitudinal division. Other factors besides failure of chromosomes to pair and poor disjunction of quadri-

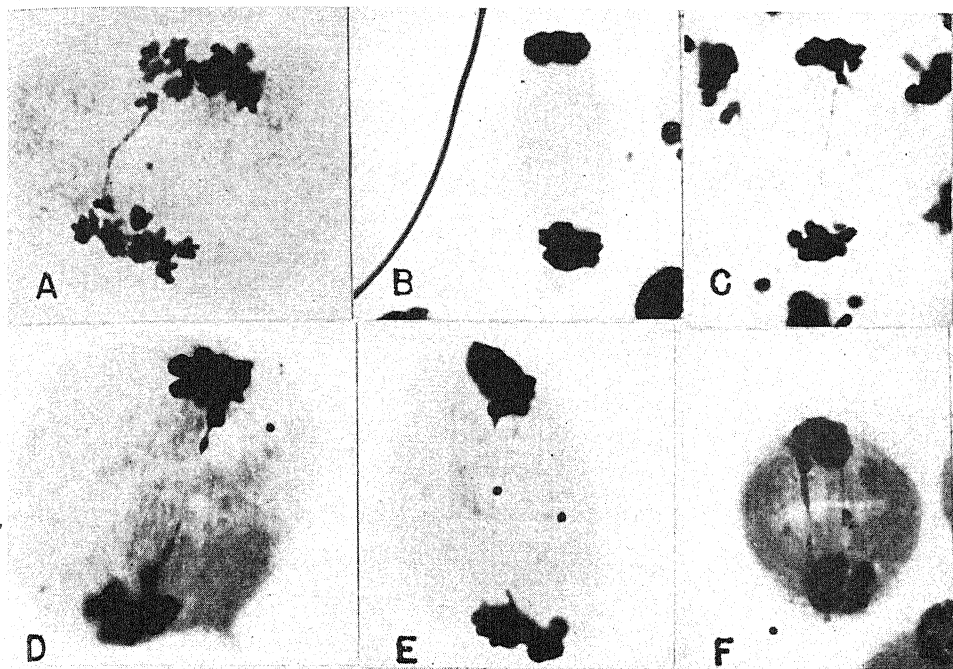


FIG. 2.—A, chromatid bridge and fragment at anaphase I; B, fragment at telophase I; C, D, E, chromatid bridges and fragments at telophase I; F, double chromatid bridge and single chromatid bridge at interphase I.

valents may be involved in the origin of lagging chromosomes. In one diploid plant of *Lolium perenne* L. the senior writer (unpublished) found a frequency of lagging univalents at anaphase I much too great to be explained on the basis of unpaired chromosomes at metaphase I. Quadrivalents were not found in this plant.

One of the striking features of anaphase I in the plants used in this investigation was the occurrence of chromatin bridges and acentric fragments (fig. 2A). These configurations appeared to be identical with those described by McCLINTOCK (4, 5, 6) and others as arising from crossing-over within an inversion. It

may be seen from table 4 that these configurations were observed in all but two plants, and in these plants only three and sixteen anaphase figures, respectively, were examined. In these same two plants telophase I nuclei clearly showed bridges and fragments. Crested wheatgrass 4(4) had some anaphase I figures with two bridges and two acentric fragments, indicating that they may have been heterozygous for two inversions. In tall oat grass 12(1) the high percentage of sporocytes

TABLE 4  
PERCENTAGE OF ANAPHASE I SPOROCYTES SHOWING ONE AND TWO DICENTRIC BRIDGES AND ACENTRIC FRAGMENTS, AND PERCENTAGE SHOWING ONE ACENTRIC FRAGMENT

PLANT NO.	No. EXAMINED	BRIDGE+FRAGMENT		FRAGMENT
		1	2	1
	ORCHARD GRASS			
	11(13).....	55	3.6	.....
	22(2).....	48	10.4	.....
	138(3).....	16	.....	.....
	CRESTED WHEATGRASS			
	4(1).....	84	14.3	.....
	4(4).....	60	16.7	1.7 3.3
	4(6).....	11	27.3	18.2
	9(7).....	3	.....	.....
	TALL OAT GRASS			
	4(4).....	88	4.5	2.3
	9(7).....	60	1.7	.....
	12(1).....	168	41.7	10.1
	12(3).....	135	2.2	.....

showing bridges and fragments suggests that it also may be heterozygous for more than one inversion, or that a long inversion permitting frequent crossing-over is involved. The assumption of heterozygosity for more than one inversion is supported by the occurrence of two bridges plus two fragments in one nucleus at telophase I.

INTERPHASE I.—The fate of the lagging and dividing univalents during the first division of meiosis can be determined approximately from the frequency of micronuclei found in the sporocytes at interphase I. Observations of late ana-

phase I and early telophase indicated that some of the half chromosomes resulting from the equational division of univalents were being included in the daughter nuclei. Others apparently lagging too far behind to be included would be left in the cytoplasm to form chromatin clumps or true micronuclei. Each lagging univalent at anaphase I has the potentiality of producing two micronuclei at interphase (fig. 1J). If all laggards were left in the cytoplasm, a given plant should have approximately twice as many micronuclei at interphase as lagging univalents at anaphase I. A comparison of the data given in table 5 with those given for anaphase I of the same plants in table 3 shows that this was not the case. The majority of the half chromosomes evidently were included in the daughter nuclei, as suggested from the observations just noted. Whether laggards formed micronuclei or were included in telophase I nuclei appeared to be conditioned simply by their chance position when the nuclear membranes were formed, and this must have operated similarly in different plants, since there was close relation between frequency of micronuclei and frequency of lagging univalents for the different plants. This relation is further evidence of the significance of the differences between plants in the meiotic irregularities studied. The chief discrepancy in this general relation is found in the four plants of crested wheatgrass. Since only three anaphase I figures were examined in plant 9(1), the discrepancy here has little significance. In plant 4(4) the percentage of sporocytes showing micronuclei was much lower in relation to the frequency of lagging univalents than in 4(1) and 4(6). The data on interphase I in the former plant were taken from a slide from a single anther in which approximately 50 per cent of the sporocytes were in metaphase I. The possibility exists that the normal sporocytes in any sac undergo division more rapidly than the abnormal and that those examined from plant 4(4) were selected from the more precocious group. In addition to the sporocytes recorded in table 5 for crested wheatgrass 4(6), one very abnormal telophase I was observed. Two groups of chromosomes had formed on one side of the division wall and one group on the other. In addition there were observed six chromatin clumps, each of which appeared to have arisen from a single half chromosome, two fragments, and one chromatin bridge.

A part of the sporocytes in each plant showed fragments and remnants of broken chromatin bridges at telophase I and interphase. Figure 2B shows a fragment at telophase I, while fragments and broken bridges are shown in figure 2C, D, and E. In general the relative frequency for different plants compared favorably with the data from anaphase I reported in table 4. In all plants, however, the percentage of sporocytes showing these configurations was lower at telophase I and interphase than at anaphase, probably because of observational difficulties. Apparently the presence of the fragment at these stages was frequently obscured by its inclusion in one of the daughter nuclei. The condition of



the bridge remnants was seen to vary, from cases in which the bridge had apparently just broken (fig. 2C, D) to cases where only slight protrusions from the interphase nuclei indicated that a bridge had existed previously (fig. 2 E). No doubt some of the latter cases were overlooked. McCLINTOCK (5) reported that in maize the majority of the chromatin bridges break, the broken ends of the sister half chromatids rejoining to produce bridges in anaphase of the first micro-

TABLE 5

PERCENTAGE OF SPOROCTES AT INTERPHASE I SHOWING VARIOUS NUMBERS OF MICRONUCLEI

PLANT NO.	No. COUNTED	No. OF MICRONUCLEI IN SISTER CELLS OF SPOROCTES							No. OF MICRONU- CLEI PER 100 SPO- ROCTES
		0-1	1-1	0-2	1-2	2-2	2-3	2-4	
	ORCHARD GRASS								
	11(13).....	169	8.9	1.2	.....	.....	0.6	.....	13.7
	22(2).....	78	11.5	5.1	1.3	2.6	.....	1.3	39.9
	138(3).....	186	13.4	5.9	2.7	1.6	.....	1.1	43.9
	CRESTED WHEATGRASS								
	4(1).....	153	17.6	3.9	2.0	.....	.....	.....	29.4
	4(4).....	168	1.8	0.6	.....	.....	.....	.....	3.0
	4(6).....	184	20.6	7.6	1.1	1.1	0.5	.....	43.3
	9(1).....	109	4.6	0.9	.....	.....	.....	.....	6.4
	TALL OAT GRASS								
	4(4).....	126	4.0	2.4	.....	0.8	0.8	.....	14.4
	9(7).....	225	9.8	4.4	.....	0.4	.....	.....	19.8
	12(1).....	178	.....	1.1	.....	.....	.....	.....	2.2
	12(3).....	232	1.7	0.4	.....	.....	.....	.....	2.5

spore mitosis. On the other hand, EMSWELLER and JONES (2) found in *Allium* that the chromatin bridges did not break but disintegrated in the cytoplasm. The bridges observed in this material appeared to break in the same manner as described by McCLINTOCK (5). Studies of mitosis in the microspores in these plants have not been made. In plants showing relatively high frequency of dicentric bridges and acentric fragments, occasional double bridges (bridges consisting of two chromatids resulting from four-strand double cross-overs within the inversion) are expected. McCLINTOCK (5) reported a low frequency of such configurations. In this material, one telophase I (fig. 2F) clearly showed a double

bridge, a single chromatid bridge, and two fragments. One of the fragments was not in the same plane as the bridges.

OCCURRENCE OF MICRONUCLEI IN QUARTETS.—The frequency of chromosome loss during the two divisions of meiosis can be estimated by the percentage of

TABLE 6  
NUMBER OF QUARTETS EXAMINED AND PERCENTAGE OF VARIOUS  
TYPES OF QUARTETS WITH MICRONUCLEI

TYPES OF QUARTETS	PERCENTAGE									
	ORCHARD GRASS			CRESTED WHEATGRASS				TALL OAT GRASS		
	PLANT NO.			PLANT NO.				PLANT NO.		
	11(13)	22(2)	138(3)	4(1)	4(4)	4(6)	9(1)	4(4)	9(7)	12(3)
1.....	2.6	6.7	11.1	4.5	.....	12.3	3.5	1.4	3.3	.....
2.....	1.3	1.5	5.0	4.0	.....	12.3	0.6	2.4	2.6	.....
3.....	6.0	8.2	12.1	14.8	6.1	23.4	15.6	3.8	5.6	1.5
4.....	.....	0.5	.....	.....	.....	4.9	.....	1.0	0.3	.....
5.....	0.4	.....	3.0	.....	.....	6.2	.....	2.8	0.3	.....
6.....	.....	0.5	.....	.....	.....	.....	.....	1.0	.....	.....
7.....	.....	.....	.....	.....	.....	.....	.....	.....	0.3	.....
8.....	0.4	0.5	5.0	.....	.....	4.9	0.6	4.3	0.3	.....
9.....	0.8	0.5	.....	.....	.....	2.5	.....	0.5	.....	.....
10.....	.....	.....	1.0	.....	.....	6.2	.....	1.4	0.3	.....
11.....	0.4	0.5	.....	.....	.....	3.7	.....	0.5	0.3	.....
12.....	.....	0.5	2.0	.....	.....	1.2	.....	0.5	.....	.....
13.....	.....	.....	.....	.....	.....	.....	.....	0.5	.....	.....
14.....	.....	.....	2.0	.....	.....	.....	.....	.....	.....	.....
19.....	.....	.....	.....	.....	.....	.....	.....	1.0	.....	.....
20.....	.....	.....	.....	.....	.....	.....	.....	1.0	.....	.....
21.....	.....	.....	3.0	.....	.....	.....	.....	.....	.....	.....
30.....	.....	0.5	.....	0.6	.....	.....	.....	.....	.....	.....
31.....	.....	.....	1.0	.....	.....	.....	.....	.....	.....	.....
32.....	.....	.....	1.0	.....	.....	.....	.....	.....	.....	.....
33.....	.....	.....	1.0	.....	.....	.....	.....	.....	.....	.....
34.....	.....	.....	1.0	.....	.....	.....	.....	.....	.....	.....
No. quartets examined...	235	195	99	176	180	81	173	210	306	205
Percentage of quartets showing micronuclei...	11.9	19.9	48.2	23.9	6.1	77.6	20.3	22.1	13.3	1.5

immature microspores showing micronuclei. All data on frequency of micronuclei were taken from preparations in which the planes of both meiotic divisions were clearly visible (fig. 1L). From table 6 it may be seen that the plants showed wide differences in percentage of quartets, which showed one or more micronuclei in at

least one cell. The origin of these micronuclei is of considerable importance. A comparison of the percentage of quartets showing micronuclei (table 6) with the percentage of sporocytes having lagging univalents at anaphase I (table 3) shows a rather close relationship between these characters. As stated previously, the lagging univalents at anaphase I divided equationally and the two halves were included in the daughter nuclei, or—less frequently—were left in the cytoplasm. Those which were included in the telophase I nuclei would be unable to take a normal part in the second division and might lag and form micronuclei in the microspores. No evidence of degeneration of the micronuclei during interphase I was obtained. If they did not disintegrate, they would persist as micronuclei in the quartets.

TABLE 7

TOTAL NUMBER OF MICRONUCLEI PER 100 QUARTETS EXPECTED ON ASSUMPTION THAT ALL HALF CHROMOSOMES RESULTING FROM LAGGING UNIVALENTS AT ANAPHASE I ARE LEFT IN CYTOPLASM AS MICRONUCLEI; AND NUMBER OF MICRONUCLEI OBTAINED

PLANT NO.	ORCHARD GRASS		PLANT NO.	CRESTED WHEATGRASS		PLANT NO.	TALL OAT GRASS	
	EX-PECTED	OB-TAINED		EX-PECTED	OB-TAINED		EX-PECTED	OB-TAINED
11(13).....	54.6	19.4	4(1).....	83.4	34.8	4(4).....	59.0	64.2
22(2).....	68.7	36.1	4(4).....	46.6	6.1	9(7).....	70.0	23.7
138(3).....	348.0	133.3	4(6).....	109.2	168.8	12(3).....	10.4	1.5
			9(1).....	132.0	25.6			














































The fact that the percentage of quartets showing micronuclei was generally lower than the percentage of nuclei showing laggards at anaphase I in a particular plant indicates that the half chromosomes are frequently included in the quartet nuclei. Since each univalent should result in two micronuclei, it should be possible to predict the total number of micronuclei expected per 100 quartets, if none of the half chromosomes are included in the quartet nuclei. These expected values and the numbers obtained are given for each plant in table 7. In eight of the ten plants, the obtained values were considerably lower than the expected, indicating that a majority of the half chromosomes must have been included in the nuclei during the second division. Of the two plants which were exceptions, the data for crested wheatgrass 4(6) may not be reliable since only eleven anaphase I figures were examined. Tall oat grass 4(4) may represent a true exception. The question arises whether micronuclei originate in this material from any source other than lagging and dividing univalents at anaphase I. It seems possible that some could arise from disturbances in the second division itself. If the excess of obtained over expected frequency of micronuclei in tall oat grass 4(4) is sig-

nificant, micronuclei must have arisen in this plant from some other source. No estimate of error is available, however, and the significance of this difference cannot be determined. Some evidence bearing on the question can be derived from the types of quartets formed by the different plants. In all observed cases, the two half chromosomes from a lagging univalent were passing to opposite poles and apparently were destined to be included in different telophase I nuclei or to be left as micronuclei in different cells of the sporocyte. If this is uniformly the case, it is possible to predict the types of quartets, based on the number and position of micronuclei, which can be produced from sporocytes with different numbers of lagging univalents at anaphase I. The expected types from sporocytes with one, two, and three lagging univalents are given in figure 3A. If neither half chromosome is included in a nucleus at telophase II, a sporocyte with one laggard would produce either type one or two. If one of the half chromosomes is included in a telophase II nucleus, type three would result. Sporocytes with two lagging univalents can produce types one to twelve inclusive, while those with three can produce types one to thirty inclusive. A plant with a low frequency of sporocytes with two lagging univalents will produce only a low proportion of types four to twelve, and not all these types would be expected unless large numbers of sporocytes were examined. The same condition would obtain for types thirteen to thirty in plants showing a low percentage of anaphase I nuclei with three lagging univalents. Obviously, if all half chromosomes are included in the telophase II nuclei, a normal appearing quartet would result. This would account for the reduction in the percentage of quartets with micronuclei in most plants below the expectation from anaphase I data.





The percentages of quartets of the various types are given in table 6. Tall oat grass 12(3) was the only plant, among those in which a large number of anaphase I figures were examined, which never showed more than one lagging univalent. As expected, this plant had no more complicated types of quartets than type three.

Orchard grass 11(13) and crested wheatgrass 4(4) and 4(6) each had anaphase I figures with one and two lagging univalents. Again, as expected, these plants showed no more complicated types of quartets with micronuclei than type twelve. A maximum of three univalents occurred in the sporocytes studied in orchard grass 22(2), crested wheatgrass 4(1), and tall oat grass 4(4) and 9(7). All except tall oat grass 9(7) had types above twelve but none had types higher than thirty. Only one plant, orchard grass 138(3), had more than three univalents per anaphase I. In this plant, four quartet types (fig. 3B) in addition to the thirty obtainable from three univalents were observed. These are numbered 31-34 in table 6. Six lagging univalents would have been necessary to account for type thirty-four. Of the sporocytes examined in this plant, 6.2 per cent of the ana-

phase I figures showed six univalents. These results on types of quartets formed by different plants are in agreement with the premises outlined and indicate that,

NO. LAGGING UNIVALENTS AT ANAPHASE I	ULTIMATE RELATIONSHIP OF LAGGING HALF CHROMOSOMES TO MICROSPORE NUCLEI	
	NONE INCLUDED	ONE OR MORE INCLUDED
1	  1      2	 3
2	    4      5      6      7	      1      2      3      8      9      10    11      12
3	    13      14      15      16    17      18	      1      2      3      4      5      6        7      8      9      10      11      12        19      20      21      22      23      24        25      26      27      28      29      30

A




  
 31      32      33      34

B

FIG. 3.—A (1-30), types of quartets (based on number and position of micronuclei) expected from sporocytes with 1, 2, and 3 lagging univalents at anaphase I, on assumption that micronuclei arise only from loss of half chromosomes derived from lagging univalents; B (31-34), four types of quartets obtained in orchard grass 138(3) which are not expected from sporocytes with 1, 2, or 3 lagging univalents at anaphase I.

in this material, most—if not all—of the micronuclei found in the quartet cells resulted from loss of half chromosomes originating from lagging univalents at anaphase I.

### Discussion

The frequency of aneuploid plants of orchard grass found in this study is somewhat higher than that reported by MÜNTZING (9), who found 10 per cent on the basis of "accurate values" and 19 per cent on the basis of "total values." It is possible that the difference between MÜNTZING's results and those obtained in this investigation can be accounted for, in part at least, by the parentage of the plants. Four of the progenies studied by MÜNTZING were from single isolated mother plants known to have  $2n=28$ . The parentage of the other four progenies was unknown, but the percentage of aberrants in both groups of four was the same. The plants used in the present study came from open pollinated seed stocks and therefore some of the parent plants were probably aneuploid. It seems logical to suppose that the frequency of aneuploidy should be higher among the progeny of aneuploid than of euploid plants. The writers are unaware of previous reports of aneuploid plants in tall oat grass. In crested wheatgrass, PETO (12) reported one plant with  $2n=29$ .

The quadrivalent frequencies in the three species investigated were almost the same. The results obtained for orchard grass agreed closely with those reported by MÜNTZING (9), who found an average of 3.8 quadrivalents per sporocyte in polar views of metaphase I in this species. Similar values were found by VON BERG (1) for *Hordeum bulbosum*, by PETO (13) for an induced autotetraploid barley, and by the writers (unpublished) for induced autotetraploids in perennial ryegrass. This similarity in the quadrivalent frequency for the different autotetraploid species of Gramineae with  $n=7$  suggests some common determining factor. The quadrivalent frequencies found in tall oat grass and crested wheatgrass do not agree with those reported by other workers. KATTERMANN (3) found 4 to 7 quadrivalents in tall oat grass and PETO (12), in reporting studies of tetraploid crested wheatgrass, stated that "pollen mother cell preparations on some of this material showed 14 bivalents in the heterotypic metaphase." In the plants studied by the writers, no nuclei with 14 bivalents were observed.

It has been suggested that some species which now behave cytologically as allopolyploids may have been derived from autopolyploids by a process of chromosomal differentiation and selection such that eventually only bivalents are produced at metaphase I. The presence of inversions, indicated by the chromatin bridges and fragments observed in all plants used in this investigation, would be a valuable aid in such a process of differentiation. Inversions have been found generally to cause great reduction in crossing-over throughout the chromosome arms in which they occur. Even if such inhibition of crossing-over in the normal chromosome segments is not universal, very little effective crossing-over within the inverted area would occur, since all single cross-overs would result in deficiencies and would probably therefore be inviable. Such inhibition of recombina-

tions resulting from crossing-over would cause an accumulation of genic and chromosomal changes in the chromosome arm containing the inversion and thus gradually result in differentiation which would reduce homology. The different quadrivalent frequencies shown by the plants investigated suggest that some selection toward bivalent instead of quadrivalent formation may already have occurred, but it does not appear to have proceeded very far. Many plants of these three species, in addition to those reported in this paper, have been examined, and in all cases quadrivalents were found in meiosis. This suggests that selection for reduced quadrivalent frequency has not been an important factor in these species or that the species are relatively young from the standpoint of number of sexual generations. Both factors might be operating. In perennial species, the selection for the higher fertility resulting from more regular meiosis would be less important than in annual types dependent entirely upon seed production for survival. Also perennial species would not be dependent upon a sexual generation each year as are annuals. This may account, in part at least, for the fact that the only species of Gramineae which have been reported to behave cytologically as autopolyploids are perennial in growth habit.

Wide differences were found for the plants studied in each of the meiotic irregularities recorded, namely, univalents at metaphase I, lagging and dividing univalents at anaphase I, and presence of micronuclei at interphase I and in the quartets. The magnitude of the differences suggests that they are significant. Further, the high correlation of these irregularities in the different plants suggests that there is a relationship among them and that the differences are conditioned by heritable factors rather than by environmental fluctuations. POWERS (14, 15) found a correlation between meiotic irregularities in varieties of *Triticum aestivum*, and MYERS and POWERS (11), using presence of micronuclei in the quartets as a measure of meiotic instability, demonstrated that heritable differences for this character occurred in these varieties.

The data presented in this paper yield some information on the nature of the relationship between the abnormalities studied. The correlation of frequency of univalents at metaphase I with that of lagging univalents at anaphase I indicates that probably most of the unpaired chromosomes lag at the equatorial plate. In some plants there were more lagging univalents at anaphase I than could be accounted for by the number of univalents at metaphase I, indicating their origin from some other source. The lagging univalents divided equationally and the daughter half chromosomes moved toward the two poles, either to be included in the daughter nuclei or left in the cytoplasm as chromatin clumps or micronuclei. The frequency of micronuclei at interphase I was considerably lower than expected if all the half chromosomes were left in the cytoplasm, indicating that a majority were included in the interphase I nuclei. In all but two of the plants

studied, the number of micronuclei in the quartet cells was lower than predicted on the assumption that all lagging and dividing univalents at anaphase I were destined to be left in the cytoplasm at the end of the second division. The observed number of micronuclei varied from 13 to 52 per cent of the expected. The data for one of the exceptional plants were too limited to be reliable. The other may represent a true exception. Whether the half chromosomes are capable of movement at anaphase II, or are merely included in the quartet nuclei if lying close to them, has not been determined. The possibility of origin of micronuclei from some source other than lagging and dividing univalents at anaphase I was suggested in the one plant which had more micronuclei than expected on this assumption. The significance of this excess, however, could not be established from the data available. A comparison of the types of quartets obtained with the types expected on the assumption that micronuclei arise only from dividing univalents indicated that in this material they originate from other sources rarely, if at all.

Aneuploid gametes apparently may be produced either from unequal distribution of quadrivalents or trivalents plus univalents or from the lagging and dividing univalents at anaphase I. For example, if a single univalent lags and divides in the first division and if both half chromosomes form micronuclei, there will be produced two microspores with 13 and two with 14 chromosomes; or, if both are included in the quartet nuclei, one microspore with 13, two with 14, and one with 15 chromosomes will be produced. The wide differences in univalent frequency between the plants studied suggest the possibility of selecting for a low univalent frequency at anaphase I in order to reduce the number of aneuploid gametes.

From the standpoint of practical breeding, these results are of considerable importance. Theoretically plants behaving cytologically as autotetraploids will show tetrasomic inheritance in genetic studies. Preliminary genetical data indicate that tetrasomic ratios are obtained in orchard grass. Tetrasomic inheritance greatly complicates the varietal improvement program in recombination of desirable characters, tests to determine homozygosity of plants for particular characters, or rate of approach to homozygosity in inbreeding. The occurrence of aneuploidy adds further complication, since  $F_1$  plants might be trisomic or pentasomic for the chromosome carrying the particular gene being studied. Corresponding ratios would then be obtained in  $F_2$ .

### Summary

1. The chromosome numbers of 116 plants of orchard grass were determined from root tips. Twenty-two per cent had 27 chromosomes, 59 per cent 28, 12 per cent 29, and 8 per cent 30. One plant out of ten in tall oat grass had 27



chromosomes, and one out of six in crested wheatgrass had approximately 31 chromosomes, while the rest were normal ( $2n = 28$ ).

2. In all three species the chromosomes were associated in pairs at mid-prophase of the first division, but frequent exchanges of partners indicated some quadrivalent formation. Occasionally a single pair of homologues were associated throughout their entire length.

3. The number of quadrivalents per microsporocyte varied from 1 to 7 in each species. For three plants of orchard grass the range in average quadrivalent frequency was 3.3 to 4.2 with an average of 3.9; for five plants of crested wheatgrass it was 3.4 to 4.2 with an average of 3.7; and for nine plants of tall oat grass it was 3.4 to 4.5 with an average of 3.8.

4. The percentage of ring and chain quadrivalents showing an alternate type of orientation at metaphase I varied from 61.8 to 90.7 among four plants of tall oat grass and was 79.1 and 90.6, respectively, for two plants of crested wheatgrass.

5. The plants of each species varied considerably in percentage of sporocytes with one or more univalents at metaphase I. The lowest frequency was none in 184 sporocytes examined in one plant of tall oat grass, whereas the highest frequency was 32.3 per cent in one plant of crested wheatgrass. The high proportion of sporocytes with two univalents as compared with the number with one and the morphological similarity of the two univalents in most cases suggested that two univalents plus one bivalent were frequently formed instead of one univalent and one trivalent.

6. Lagging univalents at anaphase I divided equationally and the daughter half chromosomes moved toward the two poles. The frequency of lagging univalents in different plants corresponded in general with the frequency of their metaphase I nuclei showing unpaired chromosomes. In most plants there was an excess of laggards, indicating that some originated from other sources than metaphase I univalents.

7. The number of micronuclei in the sporocytes at interphase I was lower than expected from the number of lagging and dividing univalents at anaphase I, suggesting that a majority of the half chromosomes arrived at the poles in time to be included in the daughter nuclei.

8. Chromatin bridges and acentric fragments which apparently had arisen from crossing-over in inversions were found at anaphase I, telophase I, or both stages in all plants studied. Three plants showed one or more sporocytes with two bridges plus two fragments, indicating that each may have been heterozygous for two inversions.

9. The percentage of quartets showing micronuclei was correlated in the different plants with the frequency of sporocytes showing lagging univalents at anaphase I. Observed numbers of micronuclei in the quartets from eight plants

varied from 13 to 52 per cent of the expected numbers, which were calculated on the assumption that all laggards formed micronuclei and none were included in the quartet nuclei. One plant had more than was expected and may have been a valid exception.

10. The number and position of the micronuclei in the quartets confirm the assumption that most, if not all, of them originated from lagging and dividing univalents at anaphase I. Only the types expected on this assumption were observed in any plant.

11. The magnitude of the differences between plants in meiotic irregularities (univalents at metaphase I, lagging and dividing univalents at anaphase I, and micronuclei at interphase I and in the quartets) and the correlation of the frequencies of these irregularities at different stages within the same plant suggest that these differences are significant and probably conditioned by heritable factors.

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# FLORAL INITIATION IN BILOXI SOYBEANS AS INFLUENCED BY PHOTOSYNTHETIC ACTIVITY DURING THE INDUCTION PERIOD

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(WITH ONE FIGURE)

## Introduction

Previous investigations (1, 4) have shown that photoperiodic induction in two typical short-day plants, Biloxi soybean and *Xanthium*, is dependent upon both light and dark periods of certain lengths. While the exact nature of the reactions affecting induction during either the light or the dark periods is unknown, it has been shown that the soybean requires light of at least 100 foot-candles' intensity if induction is to occur (2). This seems to indicate that in soybeans floral initiation is dependent upon the daily occurrence of a certain amount of photosynthetic activity during induction. The experiments described here were so designed that photosynthesis could be limited and the effect on floral induction observed. This limitation was accomplished in some experiments by removing the carbon dioxide from the air and in others by reducing the amount of light energy.

## Apparatus and methods

The apparatus for the experiments involving carbon dioxide was mounted on two warehouse trucks so that it could be readily moved to and from the dark-house (fig. 1). The plants employed were usually 8-12 inches in height and had three fully expanded trifoliolate leaves. They were either grown in or transferred to fern pots 8 inches in diameter and 4 inches deep. The plants were maintained in the greenhouse on 16-hour photoperiods until the beginning of the experiments. These photoperiods consisted of the natural photoperiod extended with Mazda light of 30-40 foot-candles' intensity. In most experiments each pot contained four plants. The plants and the pot were covered with a bell jar and sealed to ground-glass plates with a mixture of modeling clay and castor oil. Air was drawn through the system by means of a vacuum pump. The intake lines were so arranged that either natural air or CO<sub>2</sub>-free air could be introduced into the jar. Air from the greenhouse was passed through moist soda-lime towers to remove CO<sub>2</sub>. The incoming CO<sub>2</sub>-free air stream to each jar passed through a half-saturated solution of barium hydroxide in order to provide a constant check on the efficiency of CO<sub>2</sub> absorption by the soda lime.

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Excessively high temperature inside the bell jars was prevented by the circulation of cold water through small coils of copper tubing. The coils were about 6 inches long and 2 inches in diameter. Their ends extended through the rubber stoppers, which supported the coils in a vertical position immediately below the stoppers. The water was cooled by a portable unit refrigerator and pumped through the coils. By this system the air temperature inside the jars was maintained within about 5° F. of that in the greenhouse, except during brief periods of very bright light, when it rose a few degrees higher. When the apparatus was

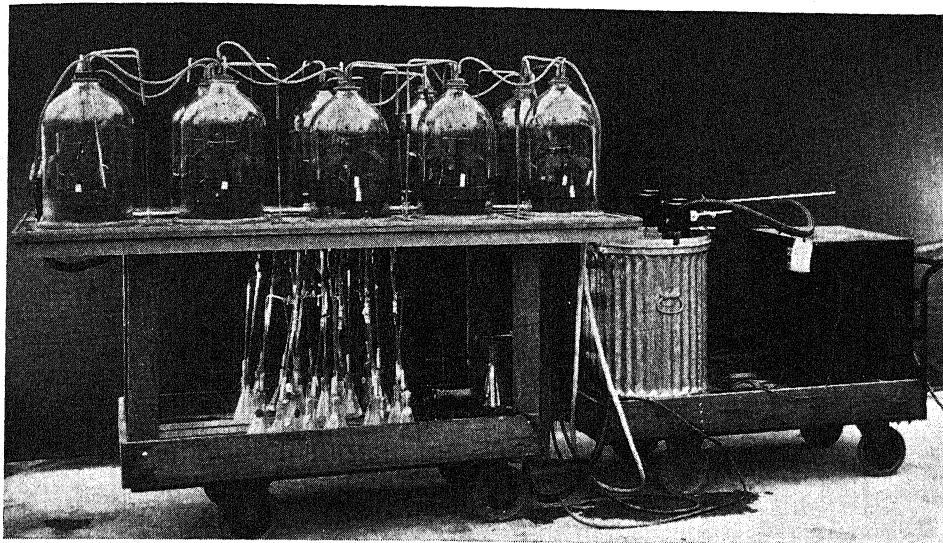


FIG. 1.—Apparatus used in experiments dealing with control of CO<sub>2</sub> in air

moved into the darkhouse, the circulation of cold water was continued for about half an hour and was then automatically stopped. Further cooling was thus prevented after the jars reached the temperature of the darkhouse. Circulation of cold water was resumed when the plants were returned to the greenhouse.

Carbon-dioxide determinations were made with either the Haldane-Henderson new model carbon-dioxide apparatus or the portable Orsat apparatus. The former was used for CO<sub>2</sub> contents of less than 1 per cent and the latter for higher concentrations.

## Results

### REDUCTION OF FLORAL INITIATION BY DECREASE OF CO<sub>2</sub> SUPPLY

A series of four experiments dealing with the effect of reduced photosynthetic activity during induction upon floral initiation was conducted during the fall and winter months of 1939 and the spring months of 1940. Control of photo-

synthesis was obtained by limiting the amount of  $\text{CO}_2$  supplied. The induction treatments in the four experiments consisted of six 8-hour photoperiods. The plants were sealed under the bell jars at about 4 P.M. and as usual received supplementary light until midnight. All plants received natural air until 8 A.M. the following morning, which was the beginning of the first 8-hour photoperiod. At this time two of the ten jars were shifted to  $\text{CO}_2$ -free air just before the trucks were moved from the darkhouse to the greenhouse. These two jars continued receiving  $\text{CO}_2$ -free air during the entire 6-day induction period. Three pairs of the remaining eight jars were shifted daily from natural air to  $\text{CO}_2$ -free air at 10 A.M., noon, and 2 P.M., respectively, during the 6-day induction treatment. The reverse shift of the six jars from  $\text{CO}_2$ -free air to natural air was made at

TABLE 1  
PHOTOPERIODIC RESPONSE OF BILOXI SOYBEANS SUBJECTED TO VARIOUS DURATIONS OF NATURAL AND  $\text{CO}_2$ -FREE AIR DURING 8-HOUR PHOTOPERIODS

TREATMENT DURING 8-HOUR PHOTOPERIOD		TOTAL PLANTS TREATED	TOTAL PLANTS BEARING FLOWER PRIMORDIA	TOTAL BUDS BEARING FLOWER PRIMORDIA PER LOT	MEAN NO. OF BUDS BEARING FLOWER PRIMORDIA PER FLOWER- BEARING PLANT
HOURS NATURAL AIR	HOURS $\text{CO}_2$ -FREE AIR				
0	8	30	0	0	0.0
2	6	30	6	6	1.0
4	4	30	17	31	1.8
6	2	26	24	58	2.4
8	0	30	24	58	2.4

8 A.M. each day after the experiment was started. The remaining jars were supplied with natural air throughout the 6-day induction period. The five pairs of jars thus received respectively 0, 2, 4, 6, and 8 hours of natural air during each of the daily 8-hour photoperiods. The treatment was continued 6 days. At 8 A.M. of the seventh day the plants were removed from the jars and returned to 16-hour photoperiods in the greenhouse. After approximately 10 days to 2 weeks, the plants were dissected. The results are summarized in table 1.

In these experiments the duration of application of  $\text{CO}_2$ -free air influenced the flowering response of the plants. When  $\text{CO}_2$  was removed from the air during the induction period no floral initiation occurred. Applications of 2 hours of natural air each day resulted in floral initiation in only a few plants, but longer treatments—particularly 6 or 8 hours—resulted in a marked increase, not only in number of plants per lot producing flower primordia but also in number of flower primordia initiated per plant.

In two experiments similar to those reported in table 1, flower primordia were formed on the plants that were scheduled to receive  $\text{CO}_2$ -free air continuously during the entire induction period. This result was caused by using barium hydroxide of too high concentration in the traps. In these two experiments the air stream to the two bell jars was frequently decreased or stopped during the dark period by accumulation of crystallized barium hydroxide in the line leading through the trap. This permitted the accumulation of  $\text{CO}_2$  from respiration of the experimental plants and soil organisms within the jar. Although the air flow was started again before removing the plants from the darkhouse, it is probable that considerable time elapsed before the  $\text{CO}_2$  was completely swept out of the jar. The presence of this  $\text{CO}_2$  is a possible cause of the formation of flower buds in these lots.

Experiments were accordingly performed to determine whether or not plants receiving no  $\text{CO}_2$  from the outside air would be able to initiate flower primordia when subjected to short photoperiods. Plants in fern pots were sealed under bell jars as in the previous experiments. When the plants were placed in the dark at 4 P.M. the flow of air to the jars was stopped by sealing the inlet and outlet lines. At 8 A.M. the plants were moved into the light and left for 2 hours before flow through the jars was resumed. Beginning at 10 A.M.,  $\text{CO}_2$ -free air was again circulated through them until 4 P.M., when they were returned to the dark. After 6 days of such treatment the plants were removed from the jars and grown for several days on 16-hour photoperiods before they were dissected. In each of two experiments of this type the plants produced more flower primordia than did control plants under jars receiving natural air at all times. Other controls receiving  $\text{CO}_2$ -free air continuously produced no flower primordia.

Since this response was apparently correlated with  $\text{CO}_2$  relationship under the jars, similar experiments were performed in which concentrations of the accumulated  $\text{CO}_2$  were determined. In these experiments an estimate of the relative amount of  $\text{CO}_2$  produced by the soil and its microorganisms and the amount produced by the plants was obtained. The apparatus was set up in the usual way except that pots containing four plants each were placed under some of the bell jars, and pots containing soil only were placed under others. As in the previous experiments, the jars were sealed at 4 P.M. and moved into the darkhouse. At 8 A.M. samples of 100 ml. of air were removed from the various jars, which remained sealed and were moved into the greenhouse at about 8:30 A.M. After 2 hours of illumination a second air sample was taken from each jar.  $\text{CO}_2$ -free air was then circulated through all the jars until 4 P.M.

At the end of the dark period the  $\text{CO}_2$  concentrations in the various jars with plants ranged from 1.5 to 1.2 per cent. The concentrations in the jars containing only pots of soil ranged from 0.7 to 0.4 per cent. The four plants evidently pro-

duced about two-thirds of the accumulated  $\text{CO}_2$  and the soil and its organisms one-third.

After 2 hours in the light the  $\text{CO}_2$  content of the air over the plants ranged from 0.7 to 0.1 per cent, depending on the intensity of the light. During this period the  $\text{CO}_2$  content of the air over the pots containing only soil increased about 0.1 per cent. During the 2 hours of light the plants assimilated an amount of  $\text{CO}_2$  approximately equal to that which they respired during the dark period. If circulation of  $\text{CO}_2$ -free air had been delayed longer than 2 hours the remaining  $\text{CO}_2$  would undoubtedly have been assimilated.

The results of these experiments show that stoppage of the  $\text{CO}_2$ -free air line during the dark period will allow the accumulation of sufficient  $\text{CO}_2$  to cause initiation.

#### REDUCTION OF FLORAL INITIATION BY DECREASE OF LIGHT ENERGY

In a series of two experiments, photosynthesis was limited by reducing the amount of light energy supplied to the plants, and its effect on floral initiation was determined. The results from previous experiments of this type (2) have shown that the extent of initiation is correlated with the amount of light energy received by the plants. In those experiments, however, the light energy was limited by reducing the intensity; in the present experiments it was limited by reducing the duration of exposure to light. This latter method made the results more directly comparable with those of the previous experiments, which dealt with reduced  $\text{CO}_2$  during the induction period.

Experiments involving decreased light energy were conducted in the greenhouse with daylight as the high-intensity source. Although the intensity of natural light fluctuated from hour to hour, it was sufficient at all times to insure active photosynthesis and was far in excess of the intensity of artificial light previously used in similar experiments (2). In the two experiments summarized in table 2, photoperiods of eight types were used. These types differed in their relative numbers of hours of high and low intensity light but were all 8 hours in duration. The high-intensity portion of these photoperiods ranged from 1 to 8 hours per day. In all cases where durations of high intensity shorter than 8 hours were used the photoperiods were extended to 8 hours with Mazda light of 10-20 foot-candles. By this intensity the dark periods to which all lots were subjected were made identical. The additional light used to bring this about was below the threshold intensity (2) previously found effective in causing initiation and constituted in all cases a very small part of the total light received by the plants.

The high-intensity part of all photoperiods was begun at 8 A.M. each day by moving the various lots from the darkhouse to the greenhouse. At hourly intervals, beginning at 9 A.M., lots were returned to the darkhouse, where they were

subjected to about 10 foot-candles of Mazda light until 4 P.M. At this time the final lot was placed in the darkhouse and the Mazda light was turned off until the following morning.

In each of the experiments the various photoperiodic treatments were applied on four successive days in one series of lots and six successive days in another series. Each lot consisted of 20 plants, making a total of 160 plants per series, or 320 per experiment. The data were subjected to variance analysis, and both photoperiod and duration of treatment were found to be significant at odds of more than 99 to 1. Since there was no significant interaction between the two factors, only the totals based on the summations of both lots have been included in table 2.

TABLE 2  
INFLUENCE UPON FLORAL INITIATION OF VARIOUS AMOUNTS OF LIGHT  
ENERGY RECEIVED BY BILOXI SOYBEANS DURING  
8-HOUR PHOTOPERIODS

TREATMENT DURING 8-HOUR PHOTOPERIOD		TOTAL PLANTS BEAR- ING FLOWER PRIMORDIA PER LOT OF 80	TOTAL BUDS BEARING FLOWER PRIMORDIA PER LOT	MEAN NO. OF BUDS BEARING FLOWER PRI- MORDIA PER FLOWER- BEARING PLANT	MEAN NO. OF NODES IN MAIN AXIS PER PLANT
HOURS OF HIGH-IN- TENSITY NATURAL LIGHT	HOURS OF LOW-INTEN- SITY MAZDA LIGHT				
1	7	0	0	0.0	19.1
2	6	10	13	1.3	19.7
3	5	62	104	1.7	19.7
4	4	75	205	2.7	20.1
5	3	77	282	3.7	20.2
6	2	80	313	3.9	20.4
7	1	80	351	4.4	20.6
8	0	80	395	4.9	20.9

The total number of plants per lot that formed flower primordia in response to these photoperiodic treatments is correlated directly with the number of hours of high-intensity light included in the 8-hour photoperiod. When the photoperiod consisted of 6 hours or more, all plants initiated flower primordia. With 3 hours of high intensity daily, about 75 per cent of the plants formed flower primordia, and with shorter durations of high intensity the number of plants that initiated flower buds decreased very abruptly. In the lots that received 3, 4, and 5 hours of high-intensity light daily most of the plants that failed to initiate flower primordia were those that received only 4 days of treatment. This might suggest that, had treatments with 1 and 2 hours of high-intensity light been applied more than 6 days, flower-bud formation would have been more abundant. The appearance of the plants in these two lots at the end of the 6 days showed that they



would not have survived treatments of much longer duration. Apparently they required more than 1 hour per day to maintain themselves. With 1 hour per day the stems elongated excessively and the leaves lost color. Each additional day of treatment served further to weaken the plants.

The number of buds per lot containing flower primordia and the mean number per plant on those that initiated flower buds were correlated with the number of hours of high-intensity light. Each additional hour of light from 1 to 8 hours caused an increase in number of flower buds. This was true even among those lots receiving 6, 7, and 8 hours of high-intensity light in which all plants initiated flower primordia.

Since the buds containing flower primordia occur singly at certain nodes of the main axis, it might be assumed that their number would be influenced by the rate of formation of new nodes in the main axis. The data of table 2 show that there is a progressive increase in number of nodes with increase in the total amount of light applied. This difference was less than two nodes, however, while the difference in number of buds containing flower primordia was 4.9. The production of buds containing flower primordia was evidently not limited by the rate of production of new nodes.

#### INCREASE OF FLORAL INITIATION BY SHORT DAILY APPLICATIONS OF AIR ENRICHED WITH CO<sub>2</sub>

Since it was possible to limit induction by reducing photosynthetic activity with the two methods applied, some stimulation to induction might be expected if photosynthesis could be accelerated. Such acceleration was attempted in a series of three experiments by increasing the concentration of CO<sub>2</sub> in natural air. Four different concentrations of CO<sub>2</sub> were established in the various bell jars each morning during the treatment period, before bringing the plants into the light. These higher concentrations were obtained by adding definite amounts of pure CO<sub>2</sub> to the partially evacuated chambers. The remaining vacuum was released by allowing natural air to flow into the jars. The inlet and outlet air lines to each jar were then closed and the apparatus was moved into the greenhouse. After remaining for 2 hours in the light, the air lines were again opened and CO<sub>2</sub>-free air was circulated through each jar during the remainder of the photoperiod and until 8 A.M. the next day.

In this series of experiments,  $\frac{1}{2}$ , 1, 2, and 4 liters of CO<sub>2</sub> were introduced into four different chambers. These amounts of CO<sub>2</sub> made the concentrations within the jars approximately 2.5, 5, 10, and 20 per cent, respectively, as measured by the Orsat apparatus. Variation in the volume of the jars as well as that of the pots and plants produced minor differences in these concentrations. In each experiment two chambers received natural air and two received CO<sub>2</sub>-free air during

the photoperiod. The results of the three experiments are summarized in series 1, table 3.

When plants received natural air during the photoperiod they formed an average of 2.5 flower primordia per plant, and only two of the twenty-five plants failed to produce any flower buds. Only one plant of the nineteen receiving CO<sub>2</sub>-free air initiated any flower buds, however, and this plant had flower buds at only

TABLE 3  
EFFECT ON FLORAL INITIATION IN BILOXI SOYBEANS OF CO<sub>2</sub> CONCENTRATIONS GREATER THAN FOUND IN NATURAL AIR

COMPOSITION OF AIR IN BELL JARS	PERCENTAGE CONCENTRATION OF CO <sub>2</sub> IN ENRICHED AIR AT BEGINNING OF TREATMENT	DURATION (IN HOURS) OF TREATMENT IN CO <sub>2</sub> ENRICHED AIR*	TOTAL PLANTS TREATED	TOTAL PLANTS BEARING FLOWER PRIMORDIA	TOTAL BUDS BEARING FLOWER PRIMORDIA PER LOT	MEAN NO. OF BUDS BEARING FLOWER PRIMORDIA PER FLOWER-BEARING PLANT
SERIES 1						
CO <sub>2</sub> -free.....			19	1	1	1.0
Natural.....			25	23	58	2.5
Enriched with CO <sub>2</sub> .....	2.5	2	12	12	51	4.2
	5.0	2	12	12	54	4.5
	10.0	2	12	12	50	4.2
	20.0	2	12	9	37	4.1
SERIES 2						
CO <sub>2</sub> -free.....			7	0	0	0.0
Natural.....			8	7	13	1.8
Enriched with CO <sub>2</sub> .....	5.0	$\frac{1}{2}$	8	7	18	2.6
	5.0	1	7	7	21	3.0
	5.0	2	8	8	33	4.1
	5.0	3	8	8	38	4.7
	20.0	$\frac{1}{2}$	8	8	23	2.8
	20.0	1	8	8	25	3.1
	20.0	2	8	8	28	3.5
	20.0	3	8	8	29	3.6

\* Plants received CO<sub>2</sub>-free air during remainder of each 8-hour photoperiod and accompanying dark period.

one node. When the air was enriched to 2.5, 5, or 10 per cent CO<sub>2</sub>, all the plants initiated flower buds and formed many more than were formed on plants receiving natural air in the jars. When 20 per cent CO<sub>2</sub> was used, however, only nine of the twelve plants treated formed flower primordia, but these nine formed nearly as many flower buds per plant as when lower concentrations were used.

Since no appreciable differences in floral initiation occurred when several concentrations of CO<sub>2</sub> were supplied for 2 hours, treatments with concentrations of 5 and 20 per cent CO<sub>2</sub> were applied for  $\frac{1}{2}$ , 1, 2, and 3 hours. By varying the dura-

tion of treatment in this manner a correlation between the amount of CO<sub>2</sub> received and the extent of initiation was shown. A summary of the results from two such experiments is shown in series 2, table 3. In both experiments one lot of plants received natural air and one received CO<sub>2</sub>-free air during the photoperiod. All the plants that received air enriched with CO<sub>2</sub> during part of the photoperiod were given CO<sub>2</sub>-free air during the remainder of each photoperiod and the following dark period.

The air that was enriched to 5 per cent CO<sub>2</sub> had a greater effect on induction than air that was enriched to 20 per cent CO<sub>2</sub>. This difference was not present in the  $\frac{1}{2}$ - and 1-hour treatments but became conspicuous as the duration was extended to 2 or 3 hours. In another experiment in which 20 per cent CO<sub>2</sub> was used for periods longer than 3 hours, definite injury to the plants resulted.

Table 3 shows that increased concentration of CO<sub>2</sub> in the bell jars caused an increase in floral initiation. Of the concentrations used, even the lower ones were very effective. Thus a 5 per cent concentration supplied for only  $\frac{1}{2}$  hour per day, or a 2.5 per cent concentration supplied for 2 hours per day, resulted in the formation of more primordia than were formed on controls receiving natural air continuously.

These results also offer a possible explanation of why more flower primordia were formed on plants that had been sealed in bell jars during the dark period and for the first 2 hours of the photoperiod than on plants that had received natural air in the jars at all times. The concentrations of CO<sub>2</sub> that accumulated under those conditions were found to be 1.5 to 1.2 per cent. While these concentrations were lower than those reported in table 3, they were probably sufficient to stimulate the production of more flower primordia than were formed on controls receiving natural air.

In several experiments plants receiving natural air under bell jars were compared with similar plants receiving natural air in the greenhouse without bell jars. In all such cases the latter plants produced many more flower primordia than the former, although the photoperiodic treatments were otherwise identical. Factors that could account for these differences include reduction of the light intensity, higher temperature, and a possible deficiency in CO<sub>2</sub> under the jars.

To eliminate the possibility of CO<sub>2</sub> deficiency, bell jars were suspended over several series of plants so that the bottom of the jar extended down only to the top of the pots. The top opening was covered with a layer of cheesecloth. This arrangement allowed greenhouse air to circulate freely through the jars. The controls for these experiments consisted of similar plants over which no jars were suspended. In a series of four experiments a total of 79 plants had bell jars suspended over them and 79 others served as controls. These plants were given six 8-hour photoperiods, after which the jars were removed and the plants returned

to 16-hour photoperiods. After 2 weeks the plants were dissected. Of the 79 plants that had been under bell jars, 61 formed flower primordia in a total of 194 buds, or in 3.2 buds per plant. All the 79 control plants initiated flower buds in a total of 415 buds, or in 5.2 buds per plant. This formation of many more flower buds on the plants outside the bell jar was characteristic of each of the four experiments. Evidently the reduced light or higher temperature or a combination of both under the jars reduced the flower-forming stimulus.

The possibility that  $\text{CO}_2$  may also have been limiting because of inadequate rate of air flow was investigated in another series of experiments by studying the  $\text{CO}_2$  content of air entering and leaving the bell jars at different rates. Flowmeters were used to determine these rates, and samples for  $\text{CO}_2$  analysis were obtained in sampling tubes inserted in intake and exhaust lines. To insure uniform samples, the tubes were left in the line 5 minutes, at which time they were simultaneously removed and the gas immediately analyzed. When air was flowing through the jars at the rate of 45 liters per hour the  $\text{CO}_2$  content on a bright sunny day decreased from 0.03 to less than 0.01 per cent. At a rate of 60 liters per hour the exhaust air contained on the average about 0.02 per cent  $\text{CO}_2$ . When a rate of 100 liters per hour was used only slight changes in the  $\text{CO}_2$  content of air were detected. The results indicate that under the conditions of these experiments the  $\text{CO}_2$  content of natural air is not greatly depleted if flows of 60 or more liters per hour are maintained. In all the experiments reported herein, flows of 60 or more liters of air per hour were maintained. The reduction in floral initiation under bell jars seems to be caused by factors such as reduced light intensity and higher temperature, rather than by deficiency of  $\text{CO}_2$  as supplied by natural air. The number of flowers formed on plants receiving increased  $\text{CO}_2$  supply under bell jars appears therefore to be a resultant of the stimulating effect of increased  $\text{CO}_2$  concentration and the retarding effect of the jars themselves.

#### INCREASE OF FLORAL INITIATION BY CONTINUOUS APPLICATION OF AIR ENRICHED WITH $\text{CO}_2$

In the experiments dealing with short daily applications of  $\text{CO}_2$  the treated plants in all cases produced more flower primordia than the control plants under bell jars receiving natural air. In a few of these experiments an additional lot of control plants not under jars was included. These plants received the same photoperiodic treatment as the other controls. Although many more flower primordia were formed in all cases on plants receiving  $\text{CO}_2$  than on controls receiving natural air under the bell jars, the most favorable  $\text{CO}_2$  treatments induced no more flower buds than were formed on the controls that were not under the jars. It seemed probable, however, that with a more favorable duration and method of application of  $\text{CO}_2$  it should be possible to initiate more primordia on plants under the

jars than on those outside. To do this the apparatus previously used was modified so that a constant stream of air enriched with  $\text{CO}_2$  to any desired percentage could be supplied to the plants.

Four-day induction treatments were given instead of the usual 6-day periods, so that fewer flower primordia would be initiated on the controls. If the plants had received 6-day treatments it would have been possible under optimum conditions that all the meristems capable of being induced on the controls would have formed primordia. By the use of a shortened induction period, any experimental treatment that was more favorable to floral initiation than that imposed on the controls should therefore result in flower-bud formation at these additional points, and the effectiveness of such treatments could be measured.

TABLE 4  
EFFECT ON INDUCTION IN BILOXI SOYBEANS OF CONTINUOUS SUPPLY  
OF AIR ENRICHED TO 1 PER CENT  $\text{CO}_2$

TREATMENT	TOTAL PLANTS TREATED	TOTAL PLANTS BEAR- ING FLOWER PRIMORDIA	TOTAL BUDS BEARING FLOWER PRIMORDIA PER LOT	MEAN NO. OF BUDS BEARING FLOWER PRIMORDIA PER FLOWER- BEARING PLANT	MEAN NO. OF NODES PER PLANT
Control in greenhouse.....	60	56	176	3.1	17.8
Natural air through bell jar at 60 liters per hour.....	60	51	98	1.9	17.3
Air enriched to 1% $\text{CO}_2$ through bell jar at 60 liters per hour.....	60	60	201	3.3	18.2

In these experiments five of the bell jars received natural air at a rate of 60 liters per hour and the other five received air enriched to approximately 1 per cent  $\text{CO}_2$  at 60 liters per hour. At the end of the 4-day induction period the plants were returned to 16-hour photoperiods. Two weeks later they were dissected.

In all the experiments, the plants that received air enriched with  $\text{CO}_2$  to 1 per cent had more flower primordia than the controls that were not under the bell jars (table 4). Each of these lots had more primordia than the lot under jars receiving natural air. By enriching the air with  $\text{CO}_2$  it was possible to cause more flower primordia to be initiated on plants under the jars than on the controls. This stimulative effect of  $\text{CO}_2$  on initiation must have been really greater than the results indicated, since it has been shown that the jars themselves have an inhibitory effect on initiation.

### Discussion

The data of previous papers (4, 6) have indicated that in Biloxi soybean reactions during the light period and others during the dark period are both essential

to floral initiation. In the present paper data are presented showing that the effectiveness of the photoperiodic treatment in causing initiation is correlated with the amount of  $\text{CO}_2$  assimilated by the plants during the light period. The initiation of flower primordia in Biloxi soybean was limited in one case by reducing the amount of  $\text{CO}_2$  made available to the plants during induction and in another by reducing the total amount of light supplied. Both treatments resulted in limiting the amount of  $\text{CO}_2$  that the plants assimilate. Conversely the initiation of flower primordia was increased by increasing the concentration of  $\text{CO}_2$  in the atmosphere surrounding the plants. When conditions of light and  $\text{CO}_2$  supply are such that considerable photosynthetic activity occurs each day during the induction period, more flower buds are initiated than when less daily photosynthetic activity occurs.

Active photosynthesis is apparently important in the photoperiodic reaction through its effect expressed within each individual leaf, rather than through its effect on the plant as a whole. Evidence for this was found in three experiments, each of which by a different method restricted the amount of photosynthesis per plant during induction. In one experiment photosynthesis was reduced by defoliation (3), in another by limitation of  $\text{CO}_2$ , and in the third by reduction in total amount of light energy supplied. When photosynthesis was limited during the induction period by these various methods, the number of flower primordia formed per plant was found to depend upon the method used. When the leaf area of a plant was reduced to about one-fourth or less by removal of all but one of the leaves, the number of flower primordia formed in response to photoperiodic treatment was frequently as great as when all leaves were present (3). In this experiment the amount of photosynthesis per plant was greatly reduced, but the amount per leaf was probably about the same as in the undefoliated controls. If, however, the amount of photosynthesis per plant was reduced by limiting the amount of light energy or of  $\text{CO}_2$  available to the plant, the initiation of flower primordia was greatly reduced. In this case the products of photosynthesis per plant may have been about the same as in the previously described defoliated plants, but the amount per leaf was probably much lower. This seems to indicate that the individual leaves must accumulate an amount of photosynthate beyond a certain threshold value before reactions will proceed that ultimately result in initiation.

It is generally accepted that the reactions responsible for floral initiation arise in the leaf (2, 5). Evidently the products of such reactions migrate from the leaves to the growing points, and there cause the formation of flower primordia. These flower-forming substances may be either immediate products of photosynthesis or products derived from them. In either event it should make no difference whether they were supplied to the growing points in small amounts by several leaves or in

larger amounts by one leaf. Since flower-bud formation occurs more abundantly in the latter case, the terminal must be receiving more flower-forming substances. The production of these substances must therefore depend on an adequate supply of the products of photosynthesis in the individual leaf and must arise through their transformation.

### Summary

1. Initiation of flower primordia in Biloxi soybean was limited by controlling photosynthesis during induction. This was done in one case by controlling the  $\text{CO}_2$  supplied to the plants and in another by controlling the duration of high-intensity light.

2. When no  $\text{CO}_2$  was supplied during 8-hour photoperiods, no initiation of primordia occurred. Plants that received the  $\text{CO}_2$  contained in natural air during 2, 4, 6, and 8 hours of each photoperiod produced flower primordia in proportion to the duration of time the natural air was supplied.

3. Plants that received only 1 hour of high-intensity light during an 8-hour photoperiod formed no flower primordia. Those that received 2 hours or more of high-intensity light during the photoperiod produced increasingly larger numbers of flower primordia as the duration of high-intensity light increased.

4. Increased concentration of  $\text{CO}_2$  in natural air resulted in increased floral initiation.

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# LENGTH OF DAY AND TEMPERATURE EFFECTS IN *RUDBECKIA*<sup>1</sup>

A. E. MURNEEK

(WITH ELEVEN FIGURES)

## Introduction

The two major aspects of photoperiodism in plants would seem to be the modification in size and form of vegetative development and the acceleration or delay of sexual reproduction. While both of these general effects of length of day have been widely observed and described by GARNER and ALLARD (5, 6) and subsequently by many other investigators of photoperiodism, none seem to have studied more closely the relationship, or absence of it, between photoperiodic inhibition and photoperiodic induction (for terminology see 11).

A previous paper (10) has shown that the influence of length of day on stem elongation and on flowering are two separate phenomena, although brought about by the same causal external factors (1). By a certain combination of photoperiods, *Rudbeckia* plants of either natural size or with extremely reduced or inhibited stem development (rosettes) were made to produce flowers normally. The present paper deals with a further study of photoperiodic inhibition and induction in *R. bicolor* var. *superba* and *R. speciosa* Newmanii (Wenderath), the latter species being used in a general way only.

## Material and procedure

Under ordinary greenhouse conditions the seeds of *Rudbeckia* germinate promptly, the seedlings are easy to transplant, and they grow slowly. This comparative sluggishness in their development makes them less sensitive to minor alterations in the external factors, which is often highly desirable when the greenhouse environment is only partly under control.

Seeds were secured from a reliable commercial source, and the germinal variability within the species was slight. During the 2 years that this investigation was in progress the treatment of the seedlings and mature plants was identical. They were grown in good garden soil in 5-inch clay pots.

Plants exposed to short days were given 7 hours of light between 8:00 A.M. and 3:00 P.M., while those receiving long days were given 14 hours of light, when necessary the daylight being extended by supplementary electric lighting of about

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250 foot-candles. The general greenhouse equipment has been described elsewhere (11). In almost all instances the experimental treatments were repeated; hence the results are drawn from duplicate groups of plants grown in different years.

## Results

### BEHAVIOR UNDER NATURAL SHORT AND LONG DAYS

When grown at an average day temperature of  $70^{\circ}$ – $75^{\circ}$  F. and approximately  $5^{\circ}$ – $10^{\circ}$  lower at night, under a natural length of day between November and July, at Columbia, Missouri (10.5 to 14+ hours of photoperiod), *Rudbeckia* plants behaved as follows. The seedlings developed slowly during the short days of the late fall and winter months into an extremely flat rosette until about the middle of March, when the daylight had lengthened to 12 hours. The leaves then assumed a more vertical position (fig. 1), which seems to be the first sign of change in development—most probably induction of the reproductive phase. This was followed about 2 weeks later by rapid elongation of the main stem and axillary shoots, until in late spring a height of 85–95 cm. was attained, when the plants were in full bloom (fig. 2, left). The critical light period (photoperiod) for initiation of reproduction in *R. bicolor* is therefore approximately 12 hours or slightly more (17). It may be the same for *R. speciosa*, although this has not been definitely established.

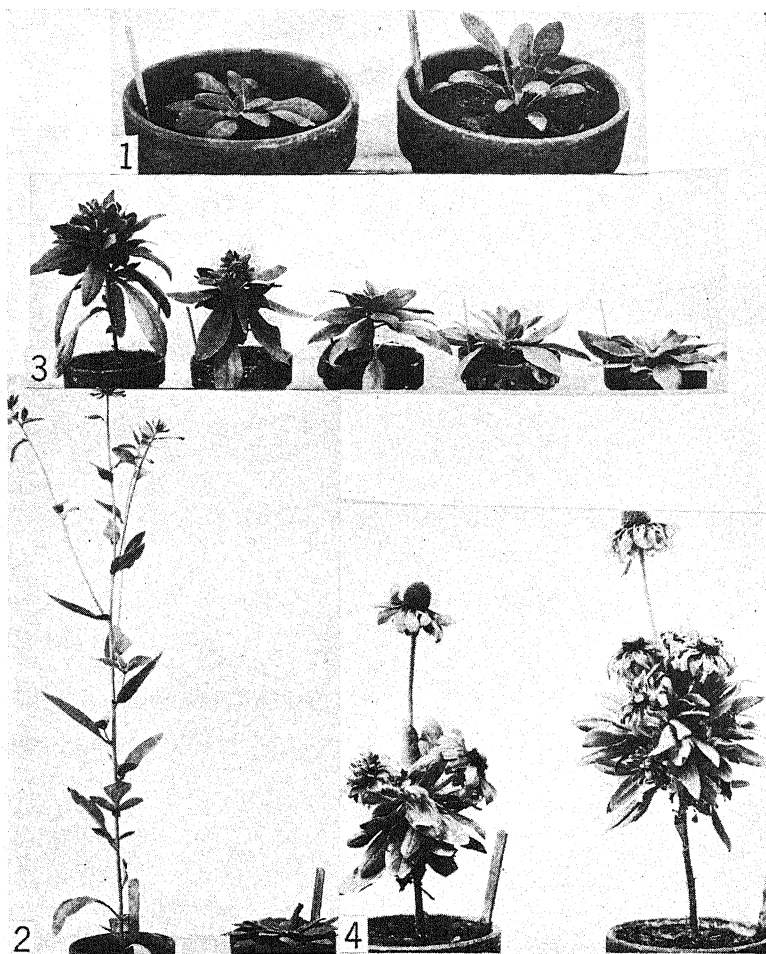
If from the seedling stage on, the plants are exposed continuously to long photoperiod (14-hour day), development is quite similar. Naturally the rosette stage is then comparatively brief or almost absent. While long days remarkably hasten development from seed to seed, the final height and form are the same as when plants are grown under natural length of day. *Rudbeckia* is a long-day plant. When exposed continuously to a relatively short photoperiod of less than 12 hours, the plants remain in the rosette stage indefinitely (fig. 2, right), some having been maintained in this form for more than a year. Neither do they grow in height nor become reproductive, provided the temperature is not extreme. At any time, however, they may be made to develop normally by moving them to long-day exposure, photoperiodic induction beginning in every case on the second day, as indicated by change in position of leaves. Retardation in development is not permanent, therefore, no matter how long continued.

In general, a relatively short photoperiod of less than 12 hours inhibits both reproduction and stem elongation in *Rudbeckia*, and a photoperiod exceeding 12 hours leads to induction of reproduction, followed shortly by growth in height of the plant.

### PHOTOPERIODIC INDUCTION AND INHIBITION

While it has previously been demonstrated in a preliminary way (10) that in *Rudbeckia* the photoperiod controls separately sexual reproduction and vegetative

growth, more extensive evidence seemed desirable as final proof of the independence of the two major effects produced by the light period.



FIGS. 1-4.—*R. bicolor*. Fig. 1, effects of length of day on position of leaves: left, grown under photoperiod  $< 12$  hours; right, under photoperiod  $> 12$  hours. Erect position seems the first sign of induction of reproduction. Fig. 2, left, grown under long (14-hour) day; right, under short (7-hour) day. Fig. 3, right to left: plants having received preliminary long-day exposure of 20, 25, 30, 35, and 40 days respectively, when moved (January 23) to short-day exposure were promptly inhibited in growth in height and looked like this a month later (February 23). Fig. 4, plants having received long-day induction of 35 days (left) and 40 days (right), respectively, inhibited in growth in height and developed some flowers normally, others subnormally or abnormally (vegetatively).

Uniform plants of *R. bicolor* that had been exposed in groups to a 14-hour day, at 5-day intervals for 20-40 days, were switched to short-day exposure (on Jan-

uary 23). Because of the preliminary long-day treatment, photoperiodic induction to various degrees had been brought about in these plants, although as yet there were no visible flower buds. The plants varied in height from 2 to 20 cm. but were extremely uniform in this respect within each subgroup (table 1, group 1).

TABLE 1

PHOTOPERIODIC INHIBITION IN *R. BICOLOR*: DEMONSTRATION BY MEANS OF SWITCHOVER EFFECTS FROM LONG- TO SHORT-DAY EXPOSURES

GROUP 1: LONG → SHORT DAYS, ON JANUARY 23

PRELIMINARY EXPOSURE		HEIGHT OF PLANTS (CM.), LONG→SHORT			HEIGHT OF PLANTS (CM.), LONG CONTINUOUS (CHECKS)	
BEGINNING OF LONG-DAY EXPOSURE	NO. OF DAYS UNDER LONG PHOTOPERIOD (TO JAN. 23)	JAN. 23, DAY OF SWITCHOVER	FEB. 25	APR. 6	JAN. 23	APR. 6
Dec. 13.....	40	20	20	21	20	90
Dec. 18.....	35	15	15	16	15	90
Dec. 23.....	30	8	8	8	8	90
Dec. 29.....	25	3	3	3	3	90
Jan. 3.....	20	2	2	3	2	90

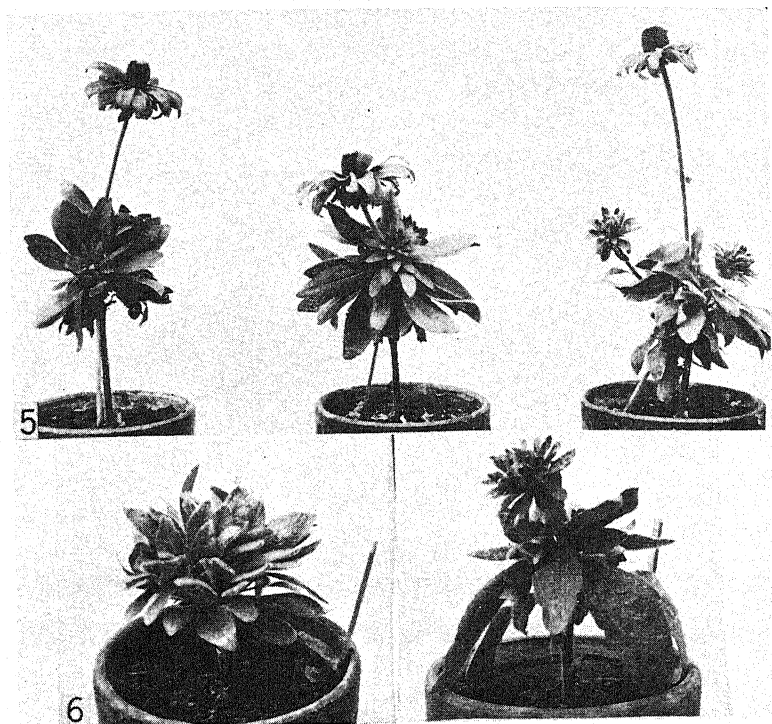
GROUP 2: LONG→SHORT DAYS, ON MARCH 1

PRELIMINARY EXPOSURE		HEIGHT OF PLANTS (CM.), LONG→SHORT			HEIGHT OF PLANTS (CM.), LONG CONTINUOUS (CHECKS)		
BEGINNING OF LONG-DAY EXPOSURE	NO. OF DAYS UNDER LONG PHOTOPERIOD (TO MAR. 1)	MAR. 1, DAY OF SWITCHOVER	MAR. 25	APR. 16	MAR. 1	MAR. 25	APR. 16
Jan. 27.....	31	34	36	36	34	80	90
Feb. 2.....	26	23	24	24	23	63	90
Feb. 7.....	21	11	11	11	11	50	90

As early as 2 days after they had been subjected to short photoperiod, plants of the December 29 and January 3 subgroups had turned their leaves into a more horizontal position, while those receiving a more extended preliminary long-day treatment did not exhibit this movement. But the most striking effect of the long → short-day plants was their prompt inhibition of growth in height. On February 25, a month after they had been switched, the height was still the same, and on April 6, when most of them were beginning to bloom, it had not noticeably changed.

While their height did not alter under short photoperiod, additional leaves

were produced in abundance, indicating that all axillary shoots had formed rosettes of foliage, giving an extremely bunched appearance to the plants (figs. 3-5). The usually long pedicels of the flowers were also mostly (but not always) abbreviated (fig. 4). Plants receiving the minimal preliminary long-day treatment (20 or 25 days) produced either one sessile flower or none at all (fig. 6), and such flowers were usually abnormal. Evidently photoperiodic induction of



FIGS. 5, 6.—*R. bicolor*. Fig. 5, vegetative flowers and vegetative rosettes on partly induced plants. Fig. 6, plants that received minimal long-day exposure (20-25 days) either did not flower (left) or produced one sessile flower (right) on short photoperiod.

reproduction in these cases was either partial or incomplete, and if complete, further development of flower parts was greatly retarded or inhibited. There were often highly "vegetative" flowers or strictly vegetative foliage rosettes present (fig. 5). The check plants, kept continuously under long photoperiods from the respective days of beginning of long-day exposure (December 13 to January 3), although varying in height considerably at the beginning (2-20 cm.), in due time grew to the same height, reaching their final growth of 90 cm. and becoming fully and normally reproductive (fig. 7).

Another group of plants was given the same experimental treatment in late

spring, when the greenhouse temperature was higher. The results were essentially the same (table 1, group 2). The slight increase in height of 1-2 cm. in some cases

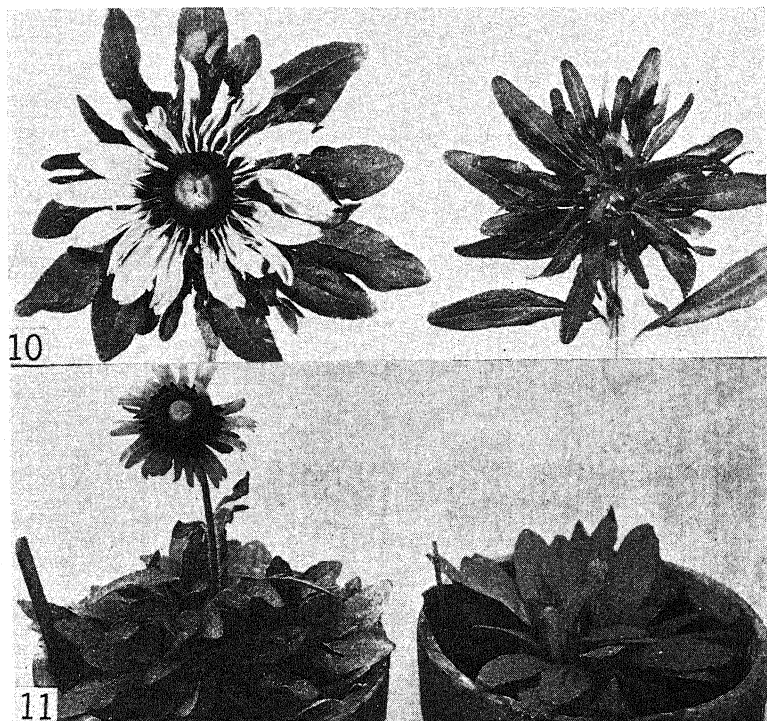


FIGS. 7-9.—*R. bicolor*. Fig. 7, although varying in height (2-20 cm.) when moved to long-day exposure, plants eventually attained same stature and uniform development. Fig. 8, flowering brought about under short (7-hour) photoperiod by relatively high temperature. Fig. 9, high temperature may induce flowering under short days, with frequent stretching of pedicels.

may have been brought about through secondary stimulation at the time of flower development (9, 11, 12), or it may have been due to an expected lag during the period of adjustment from long to short photoperiods.

## EFFECTS OF HIGH TEMPERATURE

Not being provided with refrigeration, the temperature in the greenhouse—despite proper ventilation—frequently became very high during the summer months. When the day temperature had been rising for a protracted period to 90°–100° F., occasionally higher, and the nights were proportionally warm, an unexpected thing happened. The short-day rosette type plants, which had been con-



FIGS. 10, 11.—Fig. 10, vegetative flowers of *R. bicolor* produced as result of exposure on short photoperiod to relatively high temperature (constant at 90° F.). Fig. 11, *R. bicolor* plants 250 days old (left), grown continuously under short photoperiod, came into bloom as result of high temperature. *R. speciosa* plants 250 days old (right) did not flower under same treatment.

tinuously and still were under a 7-hour photoperiod, began to bloom. In all instances only one central flower was produced, but it was usually normal in size and fully developed. Although most of the pedicels of these flowers were short (fig. 8), some had stretched and thereby elevated a few of the bractlike leaves (fig. 9), possibly due to stimulation by the flower itself (9, 12). In several cases the flowers were extremely large or highly vegetative (fig. 10). After considerable time a vegetative rosette-like flower would occasionally change into a usually subnormal flower with petals and reproductive organs. Otherwise these short-day

high-temperature plants remained unchanged in their rosette form, except for the typically more erect position of the leaves. Under long photoperiod, *R. bicolor* plants grew tall and developed normally. These results were obtained during two summers and therefore may be considered typical.

To check somewhat more closely the temperature factor, groups of plants were exposed in midwinter, when the days are naturally short, to constant temperatures of 50°, 70°, and 90° F.<sup>2</sup> After 2 days' sojourn in this uniform environment, plants in the 90° chamber had their leaves lifted and, although not growing in height, became sexually reproductive early in the spring. At uniform temperature of 70° there was also some indication of flower development, but none at 50° F. Unfortunately these artificial conditions did not imitate the fluctuating night and day temperatures of natural summer weather. It suggests, however, that probably it is the relatively high night temperature that is conducive to sexual reproduction (but not growth) of *R. bicolor*.

Although *R. speciosa* is also a long-day plant and in general responds to photoperiods of 14 and 7 hours' duration, very much like *R. bicolor*, it does not seem to be similarly sensitive to high temperature. When the two species are grown side by side on short photoperiod, they remain indefinitely in the rosette state of development, but the former does not bloom under a high temperature in June and July while the latter produces flowers (fig. 11). It would seem that *R. bicolor*, a more southern species, is "attuned" in respect to sexual reproduction to both relatively long days and high temperature, while *R. speciosa*, a more northern species, is influenced in this respect only by long photoperiod and apparently not by high temperature.

### Discussion

The present study has demonstrated that—in some plants at least—the influence of length of day on vegetative growth and on reproduction are separate responses, and that temperature may replace the photoperiod in induction of sexual reproduction.

The conclusion from the results with *R. bicolor* would seem to be as follows: (a) Growth in height is fostered and maintained, but not induced, under long-day exposure; hence when plants are placed on short photoperiod, growth of the stem and branches ceases. (b) Reproduction is not only brought about by long days but also induced. Photoperiodic induction having taken place, flower development will continue although the plants be placed under short-day exposure—a light environment nonconductive to sexual reproduction in this species. (c) Photoperiodic induction and photoperiodic inhibition, two separate responses, can be combined in various ways by exposure to appropriate lengths of day.

<sup>2</sup> The special temperature-control greenhouse was made available through the courtesy of Dr. E. BROWN of the Field Crops Department, University of Missouri.

Why should reproduction be induced but not growth when *R. bicolor* plants are exposed to relatively long days? A definite answer to this question may be made only when more accurate knowledge of the physiological causes responsible for initiation of reproduction and for maintenance of vegetative development is available. It is possible that in specific plants the formation of growth substances is fostered by certain photoperiods and not by others, and that this is a day-to-day affair with no accumulation of reserves. Hence when plants are switched to an opposite photoperiod, a shortage ensues and growth stops promptly. Or have we to deal here with inhibitors of types suggested in recent botanical literature?

That reproduction may be induced by exposure of plants to very few appropriate photoperiods has been demonstrated repeatedly. When the exposure is minimal, but few floral primordia may be able to develop into flower buds and still fewer into flowers of normal size and function. Accordingly, when *Rudbeckia* plants, after a few days' exposure to long photoperiod, are switched to short days, the flowers are subnormal in size and form, or of so-called vegetative type (2). This would seem to indicate a quantitative relationship between the production of the flower hormone, whatever its nature, and flower development (10).

The modifying effects of temperature on photoperiodism and the direct influence of temperature on sexual reproduction have been known for some time (5, 6, 7, 18, 14, 8). In respect to initiation of flowers, some plants may be very sensitive to heat or cold (18, 4). The results obtained with *R. bicolor* would seem to indicate that, as regards sexual reproduction, this species is "attuned" not only to relatively long days but also to relatively high temperature, so that the latter, as an environmental factor, may be substituted for the photoperiod. This is in agreement with previous more general observations that flowering and fruiting in this species are favored by a combination of long days and warm temperature (17). It is probable that comparatively warm nights instead of warm days may be more effective in this respect (3), and that—with normal fluctuations of temperature—there may well be certain favorable or unfavorable relationships between relatively high and low temperatures (15).

That in respect to flowering *R. speciosa* is not so sensitive to high temperature is probably due to its more northern habitat. Apparently through natural selection, based on segregation of characters or mutations, these two species have become adjusted to different environments, while other species or varieties of *Rudbeckia* may show further variability in this respect. Horticultural types, as for example a double-flower form of *R. laciniata*, may be desirable mutations, selected not only because of type of flower but also general or specific adaptability to light, temperature, and possibly other environmental factors (16). Their responses to light and heat may be different. Specific and varietal variability of this nature undoubtedly exists in almost any group of cultivated plants.



It would seem needless to emphasize the fact that great variability exists among plants in respect to their response to relatively long and short photoperiods. The cultivated dill (*Anethum graveolens* Linn.), which the writer has studied, although appearing to be a long-day plant is really an "indeterminate" or "neutral" one. The retarded growth in height and apparent vegetativeness under short-day (7-hour) exposure is merely delay in development. In a short photoperiod it required approximately 3 months for our plants to reach full stature and anthesis, while in a long photoperiod the same height and stage of development was attained in a little over 1 month. Similarly when short-day dill plants were exposed in graded series to 1-12 long days and then returned to a short photoperiod, their subsequent speed of development was in proportion to the number of long days (1-12) received. Therefore, in reference to temporarily reduced growth of dill plants under short-day exposure, it is not permissible to speak of "photoperiodic inhibition" but only of retarded development.

### Summary

1. Growth in height occurs and sexual reproduction is initiated and maintained in *Rudbeckia bicolor* under a photoperiod exceeding 12 hours. In short photoperiod, less than 12 hours, the plants remain in a rosette type of development (photoperiodic inhibition) and do not produce flowers.

2. Reproduction may be induced (photoperiodic induction) to various extents by exposure to a certain number of long days. Floral organs continue to develop and may function normally when plants are placed under short-day treatment.

3. Stem elongation cannot be induced by but only maintained on long photoperiods. Growth in height stops promptly when *R. bicolor* plants are switched from long to short photoperiod.

4. Photoperiodic induction and photoperiodic inhibition may be combined in various ways by exposing plants to appropriate combinations of long and short days. Thereby the size and form of the vegetative parts and the amount and character of development of reproductive tissues may be changed. Vegetative flowers and leaf rosettes are produced.

5. High temperature may substitute for long days in induction of reproduction in *R. bicolor*, a more southern species, but not in *R. speciosa*, a more northern one. Stem elongation is not fostered under short days by increase in temperature.

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# PARTHENOCARPIC AND NORMAL FRUITS COMPARED AS TO PERCENTAGE OF SETTING AND SIZE<sup>1</sup>

FELIX G. GUSTAFSON

(WITH ONE FIGURE)

## Introduction

In the work on artificial parthenocarpy two questions have repeatedly been asked. Are the fruits produced artificially as large as those produced by pollination? And how does the percentage of setting compare with pollination?

In the early experiments (3) there was no accurate record kept of the number of flowers treated, nor were enough fruits measured to give accurate information as to their relative size. In the paper on parthenocarpy in holly, GARDNER and MARTH (2) state: "To all outward appearances the parthenocarpic fruits are like those developed following pollination, with the exception that, in the case of naphthalene acetic acid, the fruits continue to be much greener in color and somewhat larger in size." Their illustrations confirm this statement. In 1938, the writer published some results (4) on the rate of growth and final size of tobacco fruits when produced by potassium indoleacetate and by pollination. It was found that during the first week of development the parthenocarpic fruits grew faster than the normal ones, but the final size of the seeded fruits was greater than that of the parthenocarpic fruits. WONG (11) has also found that parthenocarpic watermelons are smaller than the seeded ones.

In an experiment with cucumbers grown in the greenhouse in the spring of 1937, it was found that the setting with 5 per cent indoleacetic acid in lanolin was 28 per cent, while the setting produced by hand pollination was 66.6 per cent. The diameter of the former fruits was 12.2 cm. and the length 32.8 cm., while the diameter of the latter was 14.9 cm. and the length 37.8 cm. The percentage of setting and the size of the fruits are thus seen to be greater when produced by hand pollination.

## Investigation

In the summer of 1938 several hundred tomato plants were grown in heavily fertilized soil at the Arcadia Farm belonging to the California Institute of Technology, Pasadena, California. John Baer tomatoes were chosen because this is the plant with which the writer has had the best success in producing parthenocarpic fruits.

<sup>1</sup> Paper from the Department of Botany and the Botanic Garden of the University of Michigan, no. 716.

Owing to the very heavy growth, it was impossible to record all fruits that were set from the different treatments, and no information was gained on the number that set. To determine the rate of growth and final size, 31 fruits produced by natural pollination, 53 fruits produced by phenylacetic acid, and 15 produced by indolebutyric acid treatment were tagged and measured at frequent intervals until the fruits were ripe or had reached a stage where there was no further increase in size. Two diameters at right angles to each other and at right angles to the axis passing through the stem of the fruit were measured.

Figure 1 shows that the rate of growth and the final size reached were almost identical for the fruits produced by pollination and by phenylacetic acid. At the beginning of the measurement the indolebutyric acid-produced fruits were somewhat larger and the final size was also a little larger than the others.

During the summer of 1939 a similar experiment was conducted at Ann Arbor, Michigan. The soil was not so rich as that near Pasadena. This time the plants were pruned to a single stem and staked so that it was a simple matter to find all the treated flowers or fruits. Three different-aged plants were used in order to determine whether age and vigor had any influence upon the setting and size of fruits produced. A fourth lot of plants was grown from seeds that had been soaked in a 0.4 per cent solution of colchicine for 3 days just before planting, and a few flowers from a commercially grown lot of the same variety of plants were also treated at the time those plants were loaded with fruits.

As is usual with this variety when grown in the field, pollen was not easy to obtain and only fifteen flowers were pollinated on July 13 and 16, all on plants of lot 2. Of these, twelve, or 80 per cent, produced fruits. On July 13, when only the first two lots of plants were in blossom, flowers were treated with 3 per cent indoleacetic and indolebutyric acids in lanolin. The results are shown in tables 1 and 2.

By July 16 all four lots of plants were in blossom and a number of flowers were treated with 3 per cent naphthaleneacetic acid. The results are given in table 2. It will be noticed from these two tables that the setting due to chemical treatment of flowers on plants of lot 2 was 90 per cent for indoleacetic, 80 per cent for

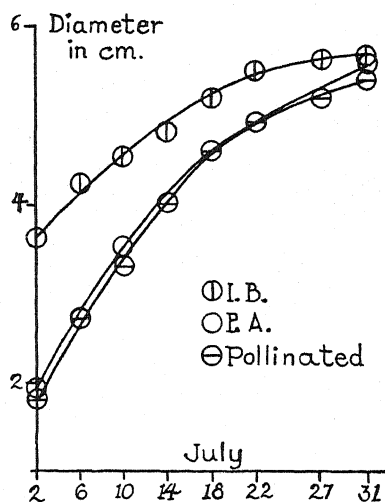


FIG. 1.—Progressive enlargement of tomato fruits produced by treating the ovaries with indolebutyric or phenylacetic acids or by pollination.

indolebutyric, and 100 and 80 per cent for naphthaleneacetic acid. This is a little better than for pollination, which was 80 per cent.

In some of the early experiments on induced parthenocarpy it had been observed that the fruit induction by chemicals was easier to obtain in the first cluster on a plant than in later clusters, after the plants had already produced

TABLE 1  
SIZE AND PERCENTAGE OF SETTING OF FRUITS FROM  
TREATED FLOWERS, JULY 13, 1939

CHEMICAL	NO. OF FRUITS PRODUCED	LOT	PERCENT- AGE SETTING	DIAM- ETER (CM.)
3% Indoleacetic.....	9	2	90	4.9
3% Indolebutyric.....	4	1	80	5.93
3% Indolebutyric.....	8	2	80	6.56

TABLE 2  
SIZE AND PERCENTAGE OF SETTING OF FRUITS FROM  
FLOWERS TREATED WITH 3 PER CENT  
NAPHTHALENEACETIC ACID

DATE OF TREATMENT	NO. OF FRUITS	LOT	PERCENT- AGE SETTING	DIAM- ETER (CM.)
7/16.....	27	1	67.5	5.44
	19	1	0*	5.97
7/13.....	5	1	46.0	5.73
	27	1	0*	6.25
	4	2	100.0	4.57
	38	2	0*	6.25
7/16.....	12	2	80.0	5.28
	8	3	80.0	6.64
	9	4	70.0	6.45
	9	4	0*	7.12

\* These fruits were seeded and had been produced by natural pollination; they are used here for the sake of comparison as to size.

some fruits. In the John Baer tomato, fruits are not readily produced in the first cluster even when hand pollinated with pollen from the same or other plants. This may indicate that there was present in the young plant a large amount of a substance necessary for the production of parthenocarpic fruits and that this substance decreased as the plants aged or produced other fruits.

GUSTAFSON and HOUGHTALING (6) have shown that when tomato vines are deflorated for part of the season they produce more flowers per cluster and the

fruits produced are much larger than those produced by plants that have been bearing during the whole season. Their interpretation is that the fruits depleted the plant's food supply and the fruits produced later in the season were small, while the fruits produced at the same time on the plants that had been deflorated were large because there had been no depletion of food substances.

These two facts made it desirable to compare old plants that had produced no fruits (due to defloration) and young plants that were just coming into flower. Plants (designated as *b* in table 3) from lots 1 and 2 were therefore deflorated for several weeks after the other plants began to bear.

TABLE 3  
SIZE AND PERCENTAGE OF SETTING OF TOMATO  
FRUITS FROM FLOWERS TREATED WITH 1 PER  
CENT NAPHTHALENEACETIC ACID, JULY 26, 1939

NO. OF FRUITS	LOT	PERCENTAGE SETTING	DIAMETER (CM.)
6.....	1 <i>a</i>	25	4.47
9.....	1 <i>b</i>	80	5.07
14.....	1 <i>b</i>	0*	5.82
2.....	2 <i>a</i>	20	5.15
4.....	2 <i>b</i>	56	6.16
16.....	2 <i>b</i>	0*	6.24
10.....	3	66	5.75
5.....	3	0*	5.34
9.....	4	43	6.49

\* These fruits were seeded and had been produced by natural pollination; they are used here for the sake of comparison as to size. The *a* plants were setting fruits all the time; the *b* plants were not permitted to set until this date.

On July 26 the ovaries of the flowers from the *b* plants—as well as from all the other plants—were treated with 1 per cent naphthaleneacetic acid. Unfortunately lots 3 and 4 had come into blossom by July 16 and were now producing blossoms in the second or third cluster; but by comparing the *b* plants from table 3 with the plants from lots 3 and 4 in table 2, comparable flowers and fruits are obtained. It will be seen that plants 1*b* set 80 per cent on July 26, so did the plants of lot 3, and those from lot 4 set 70 per cent on July 16. Plants 2*b* set only 56 per cent on July 26 while corresponding *a* plants had set 80 per cent on July 16. If a comparison is made between the *a* plants (those that had been bearing all season) and the *b* plants from the corresponding lots, on July 26, it will be seen that the percentage setting is considerably lower in the *a* plants, owing no doubt to depletion of food material as a result of fruit production. Comparing the *b* plants and those from lots 3 and 4, on July 16, it is clear that the percentage of setting in the older plants (that have not been producing any fruits) is nearly as large as in the young plants producing fruits for the first time; and the old plants

are just as capable of producing parthenocarpic fruits as the young plants if they have not been bearing too heavily. Commercially grown plants that had been bearing heavily all summer produced no fruits at all when treated with chemicals on July 26. Evidently production of fruits parthenocarpically does not depend upon the age of the plant but upon its nutritional level, which is dependent upon the amount of fruit previously produced by the plant, and upon its vigor.

In tables 2 and 3 are included fruits produced by open pollination. While the time of setting is not known, the fact that they were ripe and on the same plants

TABLE 4

COMPARISON AS TO SIZE BETWEEN TOMATO FRUITS PRODUCED BY CHEMICALS  
AND POLLINATION, WHEN GROWN IN SAME CLUSTER

DATE OF TREATMENT	PARTHENOCARPC					SEEDED	
	CHEMICAL AND PERCENTAGE		LOT	NO. OF FRUITS	SIZE	NO. OF FRUITS	SIZE
7/13.....	Indolebutyric	3	I	3	5.86	3	6.54
7/16.....	Naphthaleneacetic	3	I	27	5.75	3	6.11
7/26.....	Naphthaleneacetic	I	I	12	5.07	8	6.23
7/29.....	Indolebutyric	I	I	3	5.96	3	6.82
7/13.....	Indoleacetic	3	2	7	4.93	6	5.63
7/13.....	Indolebutyric	3	2	8	6.13	8	6.24
7/16.....	Naphthaleneacetic	3	2	15	5.16	13	5.97
7/29.....	Indolebutyric	I	2	2	6.56	2	6.07
7/16.....	Naphthaleneacetic	3	3	7	6.87	7	7.48
7/16.....	Naphthaleneacetic	3	4	6	6.55	6	7.16
All naphthaleneacetic.....		3	.....	55	5.81	56	6.36
All naphthaleneacetic.....		I	.....	12	5.07	8	6.23
All indolebutyric.....		3	.....	11	6.06	11	6.31

as the ripe parthenocarpic fruits justifies a comparison of their size with that of the parthenocarpic fruits. The fruits produced by pollination are larger in all instances, except on lot 3 treated with 1 per cent naphthaleneacetic acid on July 26, which are somewhat larger than the corresponding seeded fruits.

To make the comparison more definite, the parthenocarpic and seeded fruits which grew in the same cluster have been brought together in table 4. There is only one instance in which the parthenocarpic fruits are larger than the corresponding seeded ones, and here only two fruits are concerned. So far as the John Baer tomato grown in Ann Arbor, Michigan is concerned, therefore, the parthenocarpic fruits are somewhat smaller than the seeded ones. The difference in

size is not great, and undoubtedly the seedless fruits with the somewhat greater amount of pulp per fruit are to be preferred even though a little smaller. On the other hand, figure 1 shows that fruits produced by indolebutyric and phenylacetic acids at Pasadena, California, are as large if not larger than fruits produced by pollination. How can these contradictory results be reconciled? No doubt Californians would say it is due to the climate, but it is more likely due to the heavy fertilization of the soil, which resulted in greater vigor of the plants. The soil in which these plants grew was heavily fertilized with chicken manure, rich in nitrogen, just before the plants were set out. AVERY, BURKHOLDER, and CREIGHTON (1) have shown that the growth hormone content of a plant is influenced by the mineral nutrition. Lack of nitrogen especially decreases the auxin content very much. It is therefore likely that the auxin content of these tomato plants was so great that neither it nor the food substances needed for fruit growth was the limiting factor, while the plants growing in a less fertile soil may be lacking both in food material and auxin. Here the fruits with more auxin might conceivably grow larger.

The smaller size of the parthenocarpic fruits cannot be explained on the basis of difference in the size of the original ovary, as they all come from the same cluster. HOUGHTALING (7) has shown that in several varieties of tomato there was no cell division in the ovaries after anthesis; and MACARTHUR and BUTTLER (8) state: "The post-anthesis period is characterized chiefly by cell expansion, cell division being a minor factor which just suffices to maintain the epidermis, the cells of which do not expand."

The difference in size must therefore be due to the formation of smaller cells in the parthenocarpic fruits than in the seeded fruits. The smaller cell size in the parthenocarpic fruits is undoubtedly due to a lower concentration of auxin, as the food supply is the same. The writer (5) has shown that seeded tomatoes have a greater auxin concentration than have parthenocarpic fruits; the seeds are very rich in auxin. If quantity of auxin is responsible for size, and these experiments point in that direction, it would seem possible to increase the size of the parthenocarpic fruits by supplying them with more auxin. Methods for doing this will have to be developed, as it is not sufficient merely to smear more lanolin paste on the fruits.

These and other experiments on parthenocarpy indicate that the growth of fruits as a result of chemical treatment is in no way different from that taking place as a result of pollination, except that ovules do not develop. The matter of food supply is just as important in the production of both types of fruits. This is to be expected if we consider that an important function of the auxin is to mobilize the food material to the point where growth takes place, as seems to be implied by the experiments of MITCHELL and MARTIN (9) and STUART (10).



### Summary

1. The experiments, although rather limited on this point, show that the percentage of setting with chemical treatment is probably as great as with pollination. This refers only to the tomato. On the other hand, the parthenocarpic fruits are definitely smaller than the seeded fruits.

2. The early rate of growth may perhaps be a little greater in the ovaries treated with chemicals than in those pollinated, but this early rate decreases after a while, perhaps due to a lowering of the auxin concentration in them.

The writer wishes to express his appreciation to F. W. WENT, E. G. ANDERSON, J. VAN OVERBEEK, and WM. S. STEWART of the California Institute of Technology, Pasadena, California, for various courtesies and to the Directors of the Horace H. Rackham Trust Fund of the University of Michigan for financial aid.

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# POLYPLOID COTTONS OBTAINED THROUGH USE OF COLCHICINE. I. CYTOLOGICAL OBSERVATIONS IN OCTOPLOID *GOSSYPIUM HIRSUTUM*<sup>\*</sup>

A. J. T. MENDES

(WITH TWENTY FIGURES)

## Introduction

A preliminary account of the occurrence of polyploid cottons obtained through the use of colchicine has been presented before the First South American Botanical Congress (5). Individuals having  $2n=52$  and  $2n=104$  were obtained after treatment of seeds of *Gossypium herbaceum* L. ( $2n=26$ ) and *G. hirsutum* L. ( $2n=52$ ), respectively. The treatment which furnished the highest percentage of "duplicated" plants consisted in the immersion of delinted seeds in a 0.15 per cent colchicine solution for about 16 hours. Various treatments, however, such as with a solution of 0.075 per cent for 25 hours or 0.3 per cent for 48 hours, also produced polyploid plants.

The seeds of *G. hirsutum* were from a multiplication field of a commercial variety, while the seeds of *G. herbaceum* were from plants which had been selfed for many generations. While from the latter seeds uniformly abnormal plants were obtained as a result of certain treatment, several different types of abnormalities were found among the plants derived from the seeds of *G. hirsutum*. Some of the seeds did not react at all to the drug. The chromosome numbers were then determined in root tips when the plants were only  $5\frac{1}{2}$  months old; at that time they had not yet flowered.

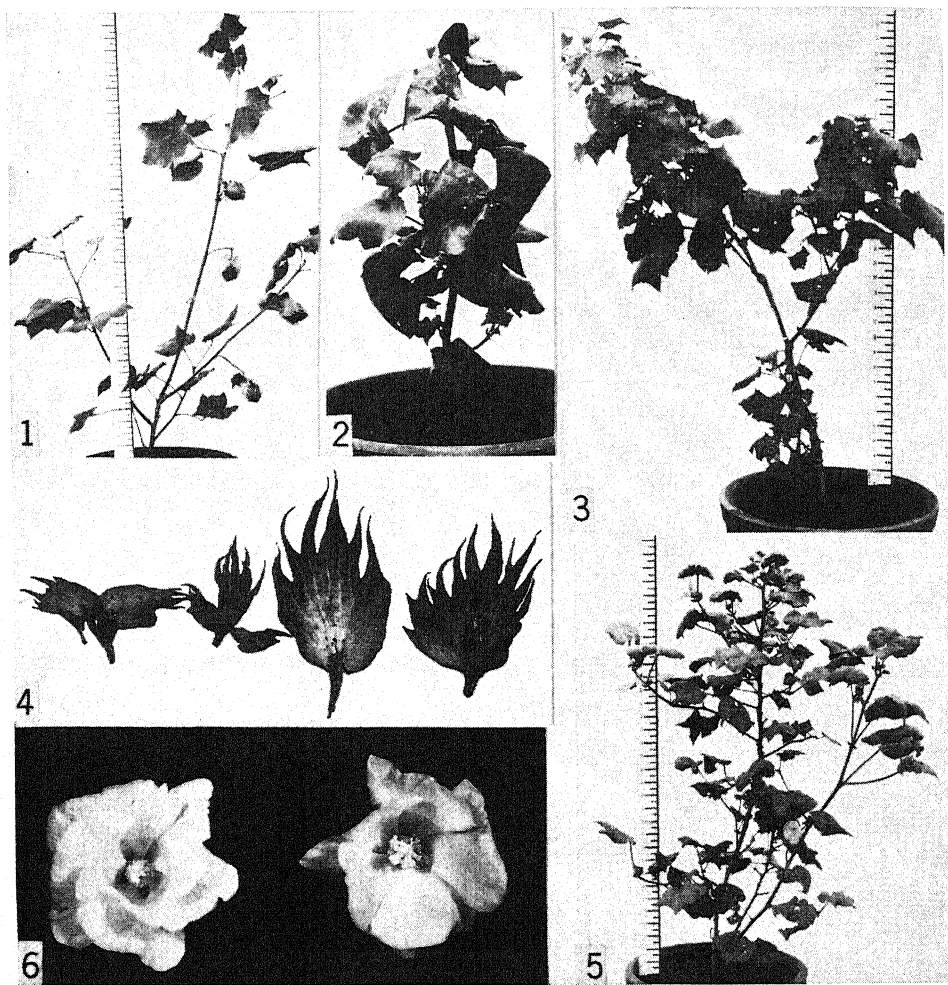
In the present paper only the experiments with *G. hirsutum* (fig. 1) will be presented, leaving for future publication the results obtained with *G. herbaceum*.

## Morphological observations

The seeds treated with low percentage (0.06) colchicine solution for a relatively long time (16 hours) did not produce other than normal plants. Seeds treated with high percentage (0.6) solution for a short time (4 hours) produced highly abnormal plants, many of them dying in the cotyledonary stage. Their hypocotyls were greatly swollen and the roots poorly developed; in the root tips, octoploid cells ( $2n=104$ ) were found. By using a moderately concentrated colchicine solution (0.15 per cent) for 16 hours both normal and abnormal seedlings were obtained; among the abnormals, several soon died. This treatment, however, has been found

<sup>\*</sup> Publication authorized by the Director of the Institute as Technical Bulletin no. 76.

to be the most effective in producing entirely octoploid plants. Both severe and moderate treatments can produce entirely octoploid plants, apparently without chimaeras; but the octoploid plants obtained through moderate treatment have a



FIGS. 1-6.—*Gossypium hirsutum*. Fig. 1,  $2n=52$ , normal plant. Fig. 2,  $2n=104$ , from heavily treated seed; no flowers. Fig. 3, same, from heavily treated seed; flowers reduced to bracts. Fig. 4, abnormal "flowers" from plant shown in fig. 3. Fig. 5,  $2n=104$ , from treated seed. Fig. 6, from two plants having  $2n=104$  and  $2n=52$ ; note indehiscence of anthers in 104-chromosome flower at left.

better chance to survive than plants produced from severely treated seeds, their development also being much more regular.

Several types of abnormalities are found among the plants from severely treated

seeds which do not occur among the others. For instance, a severely treated seed produced an octoploid dwarfed plant ( $2n = 104$  in root tips) on which the leaves were entirely different from those of all the other plants, and on which not a single flower bud has yet developed—the plant is now more than 13 months old (fig. 2). Another interesting octoploid plant (fig. 3) had its floral parts reduced to a single bract, with a reduced calyx at its base (fig. 4). The plants which appeared to be tetraploid were eliminated and about seventy of those which seemed to be octoploid were raised to maturity. Plants which proved to be entirely octoploid were rare. It was found that many of the root tips were composed of a mixture of tissues having 52 and 104 chromosomes. Later, while studying microsporogenesis, tetraploid and octoploid cells sometimes were noted in the same flower.

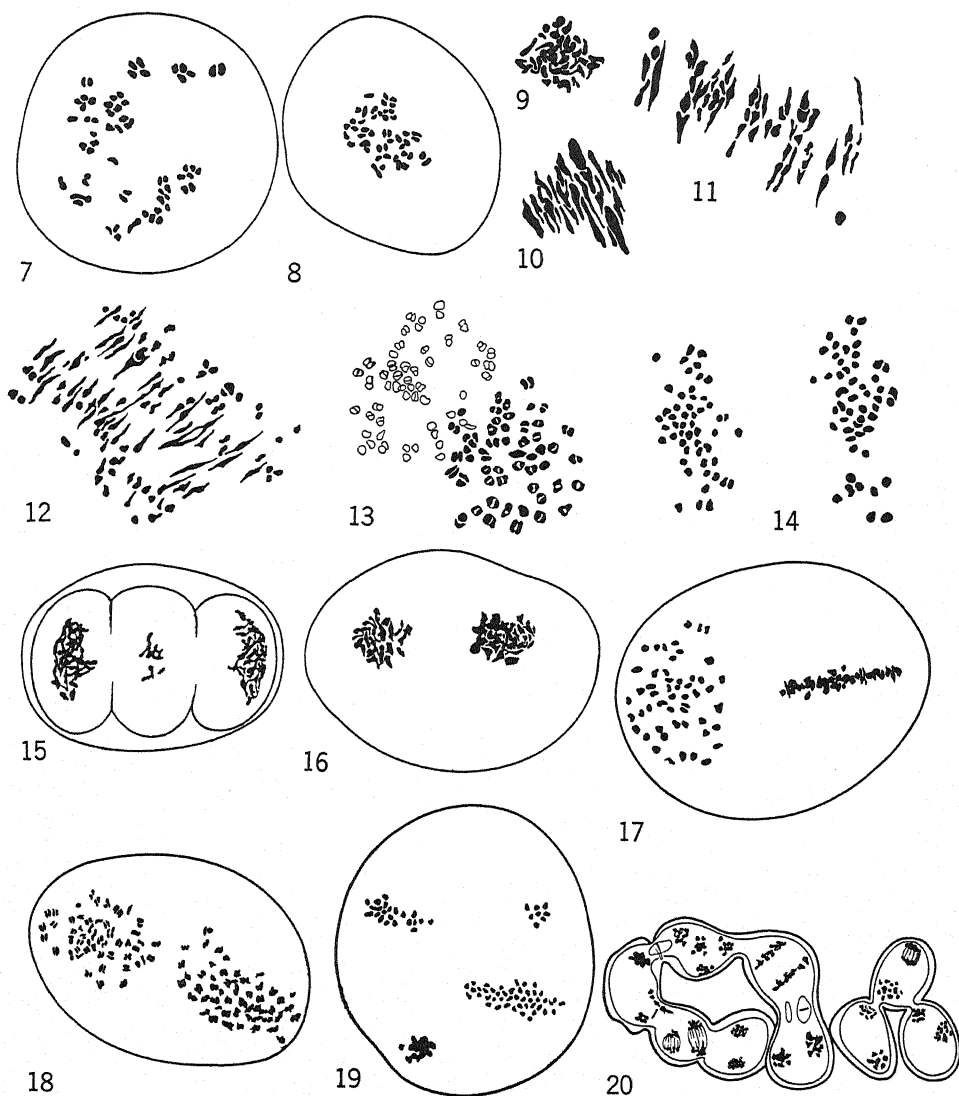
Various characters help to identify adult octoploid plants: coarse leaves of a deeper green color than in normal plants, abnormal branching and stunted growth (fig. 5), and flowers which do not open easily and have thicker petals and indehiscent anthers (fig. 6). The size of the leaves was very variable amongst the octoploids, some of them being tiny and others extremely large.

### Cytological observations

An analysis of chromosome distribution in the pollen mother cells of the octoploid plants has been made. Flower buds were collected and fixed in a mixture of 3 parts alcohol to 1 part acetic acid. After 24 hours they were transferred to 70 per cent alcohol and the anthers examined by the aceto-carmin method. All drawings, with exception of figure 20, were made with a camera lucida having a 10X ocular and a 90X objective, giving a magnification of 1070 diameters.

The prophase is difficult to observe in detail. In the thread stage the chromosomes can be seen only when the coverslip is not pressed against the material, but as soon as the coverslip is pressed down the filaments become less visible in the stained plasm. A single large nucleolus is seen in the prophase for a long time. Buds of several sizes show only prophasic cells and the next phases occur in rapid succession. In the same anther may be found cells in every stage of meiotic divisions, from prophase I to young "quartets."

The formation of quadrivalents, the separation of the halves of the bivalents, and a considerable amount of secondary association make the counting of the chromosomes difficult at metaphase I. Figure 7 shows cells in the prometaphase stage having some bivalents free and others associated in groups of two, four, and more. In figure 8 still closer association is observed. This may become so intense that massing of the chromosomes occurs (fig. 9). Close to cells like the one shown in figure 10 are others like that illustrated in figure 11, where the chromosome number could be more easily determined. The difference in the intensity of mass-



FIGS. 7-20.—*Gossypium hirsutum*,  $2n=104$ . Fig. 7, secondary association in pollen mother cells. Fig. 8, closer association of chromosomes. Fig. 9, massing of chromosomes in pollen mother cells. Fig. 10 ( $2n=104?$ ), profile view of metaphase I. Figs. 11, 12, same of metaphase I. Fig. 13, polar view of anaphase I; chromosomes already divided. Fig. 14, late anaphase I; 52 chromosomes at each pole. Fig. 15, cytokinesis after first division. Fig. 16, massing of chromosomes after first division. Fig. 17, metaphase II; 52 chromosomes in each plate. Fig. 18, metaphase II; chromosomes appear double. Fig. 19, multipolar arrangement of chromosomes. Fig. 20 ( $2n=204$ , or mixoploid), syncytia of pollen mother cells.

ing at these stages may be caused by various degrees of secondary association at earlier stages.

As already stated, the counting of the chromosomes in polar views of metaphase I is difficult. The bipartition of many chromosomes and the union of others in pairs or larger groups cause confusion; moreover, the anaphasic separation does not occur at the same time for all the chromosomes, so that univalents are often scattered away from the equatorial plate. A profile view of anaphase I is shown in figure 12, where it can be seen that some chromosomes go to the poles earlier than the others. Other anaphases were found in which the separation of the chromosomes occurred regularly and at the same time for all the chromosomes. In late anaphase almost all the chromosomes are already divided (fig. 13).

Many anaphases were observed in detail, and almost always 52 chromosomes were seen to go to each pole (fig. 14). A few pollen mother cells among normal ones from the same anther showed several types of abnormality, such as unequal distribution and losses of chromosomes between the two poles, cytokinesis after the first division, etc. This last type of abnormality has been observed only once and was a very peculiar case, the cytoplasm appearing to be divided into three portions (fig. 15).

At the end of the first anaphase a massing of the chromosomes, identical to the one observed before, can be seen (fig. 16). It seems, however, that the more general rule is the immediate formation of two second equatorial plates (figs. 17, 18). When neither massing nor immediate arrangement of the chromosomes in the metaphasic plates takes place after the first anaphase, interkinetic nuclei are formed, the chromosomes losing their compactness. Chromosomes can be easily counted at metaphase II. As in anaphase I, 52 chromosomes are distributed to each pole in the second division. Almost all the anaphases II are regular; several lost chromosomes which are occasionally observed at this stage (fig. 19) are usually laggards remaining from the first division. As a result of the predominant regularity observed in these divisions, about 73 per cent of all quartets formed really have four cells; only about 15 per cent of the microsporocytes form quartets with five cells and 12 per cent with six cells. Very seldom quartets are formed with still more microspores.

A peculiar type of abnormality, that is, the arrangement of the pollen mother cells in syncytia (fig. 20), already observed by LEVAN (3) in octoploid species of *Petunia*, was also noticed in some *Gossypium* plants. In some cases it was possible to determine their mixoploid nature, but in others it seemed certain that they were entirely octoploid. It was difficult to determine whether the syncytia were formed by tetraploid or by octoploid cells. It appeared evident that anastomosis of the cells occurred during the prophase of the first division. The two mitoses went on regularly in most cells, forming four normal telophasic nuclei, although in some

cases a shifting of chromatic matter from one cell to its neighbor cell was noticed. It is possible that this shifting occurred as a consequence of the pressure on the coverslip.

### Pollen, seed, and progeny

As already stated, indehiscence of the anthers was one characteristic of the octoploid plants. This fact is responsible for their high sterility, since the pistil always appeared to be normal and since microsporogenesis was almost completely normal. Occasionally, however, a few anthers in a flower were dehiscent. Studying the size of the pollen grains, it has been found that some anthers were diploid while others were tetraploid. The pollen grains of the untreated plants ( $2n=52$ ) had a diameter of  $106.5 \pm 5.6 \mu$ , while the ones of the treated plants ( $2n=104$ ) were generally larger and more variable,  $138.5 \pm 12.2 \mu$ . In the tetraploids the coefficient of variability was 5.5 per cent, compared with 8.8 per cent in the octoploids.

Self- and cross-pollination, using pollen from normal and treated plants (octoploid or not), has been carried out artificially, but not a single mature fruit has been obtained from more than one hundred flowers. The artificially pollinated flowers produce a fruit that does not develop very far, falling off 3-12 days after pollination. Grafts of the octoploid plants have been made on normal tetraploid ones; here also no fruit set has been obtained. Occasionally a few bolls were produced from open pollinated flowers. These rare fruits differed from the normal ones in being more nearly spherical, smaller, and few-seeded. While in the untreated plants the seeds were uniformly small and long, weighing on the average 0.73 gm., the seeds obtained from the treated plants varied in form, size, and weight. In most cases they were similar to the normal ones in form and size, weighing 0.71-0.87 gm.; in other instances the seeds were almost spherical and much larger, weighing 0.93-1.22 gm.

Some of the seeds were sown in petri dishes and after germination transferred to small pots. At the beginning only a low percentage of the larger seeds germinated, while almost all the normal ones germinated rapidly. Increased germination was later obtained by cutting off the tip of the seed coat (1). All the plants obtained were normal in appearance, flowering, and fruit set. In many of them the chromosome number was  $2n=52$ .

### Discussion

Specialists (2, 4, 6, 7) in the genus *Gossypium* have suggested that a duplication of the chromosomes of ancestral types produced the American cottons with  $2n=52$ . These are considered to be tetraploids and the plants having  $2n=104$  are thus octoploids. The secondary association of the chromosomes found in the octoploids is similar to that occurring in the tetraploids. The abnormalities found

during microsporogenesis in octoploids are slightly more pronounced than the ones reported by other workers for the tetraploids. Assuming that the same abnormalities occur at megasporogenesis as has been found at microsporogenesis, they cannot be responsible for the extremely low fruit set of the octoploids, since most of the egg cells should function normally. The high sterility of the octoploids must be due mostly to indehiscence of the anthers and other physiological disturbances.

The highly abnormal plants obtained after heavy colchicine treatments suggest changes in the structure of chromosomes or gene mutations, in addition to duplication of chromosome numbers. The facts that several seeds have been produced by the octoploid plants and that they have developed into normal tetraploid individuals might be explained either through parthenogenetic development of 52-chromosome egg cells or through the fusion of two 26-chromosome gametes both derived from tetraploid tissue islands. The second hypothesis is more probable, since such tetraploid tissue islands have actually been found both in anthers and root tips.

The different shape, the larger size, and consequently the increased weight of the seeds are to be interpreted as results of the influence of the octoploid mother plant. The gigantism of the vegetative organs, found in other known polyploids, has not been found in octoploid *Gossypium*.

#### Summary

1. Octoploid and tetraploid plants were obtained from seeds of *Gossypium hirsutum* and *G. herbaceum* treated with colchicine.
2. The seeds of *G. herbaceum* were generally homozygous and reacted as a rule more uniformly to the drug than did the seeds of *G. hirsutum*.
3. Microsporogenesis in octoploid ( $2n=104$ ) *G. hirsutum* is described in some detail: a few chromosomal irregularities were observed and the pollen grains are larger than in the normal tetraploid plants. In spite of the fact that empty pollen grains were rarely found, the plants are highly sterile. The anthers are almost always indehiscent.
4. Flowers with dehiscent anthers were self-pollinated, and others were cross-pollinated with pollen from tetraploid and octoploid plants. No fruit set occurred.
5. A few fruits from open-pollinated flowers have been obtained. Their seeds were in some cases almost spherical and were larger and heavier than the normal. The seed coat was very hard and the percentage of germination low. These seeds yielded normal tetraploids ( $2n=52$ ).

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# FORMATION OF THE SEED OF *CYPRIPEDIUM PARVIFLORUM*

MARGERY C. CARLSON

(WITH NINE FIGURES)

## Introduction

This study was undertaken to learn the steps in the formation of the seed of the orchid, *Cypripedium parviflorum* Salisb., to obtain a better understanding of the structure of the mature seed, and to obtain, if possible, some clue to the problems involved in the germination of orchid seeds in general. The history of megagametophytes and embryos in *Orchis pallens* was traced by STRASBURGER (16) and in *Gymnadenia conopsea* by WARD (19). TREUB (17) and DUMÉE (5) studied embryos of orchids, especially to determine the types of suspensors present. They found unorganized embryos in all the species studied; single-celled suspensors, which sometimes cannot be distinguished from the embryo proper, in some species; and elongated, multicellular suspensors which extend out through the micropyle in other species. BEER (1) examined mature fruits and seeds of many species of tropical orchids, and CURTISS (4) described seeds of twenty-five species native to the United States and determined their average dimensions and the dimensions of the cells of the seed coats. LEAVITT (6) described variations in suspensors and listed cases of polyembryony in several species of native orchids. VESQUE (18), PACE (9, 10), BROWN (2), BROWN and SHARP (3), and SHARP (13, 14) traced megasporogenesis, development of the megagametophyte, and fertilization in certain species of orchids; but none of these investigators followed the history of the seed beyond the first few divisions of the fertilized egg. PFITZER (11) summarized the information on seeds of orchids available in 1889; NETOLITZKY (8) described the structure of mature seeds; and MAHESHWARI (7) and SCHNARF (15) reviewed the work on megagametophytes, endosperm, and embryos of orchids.

## Methods

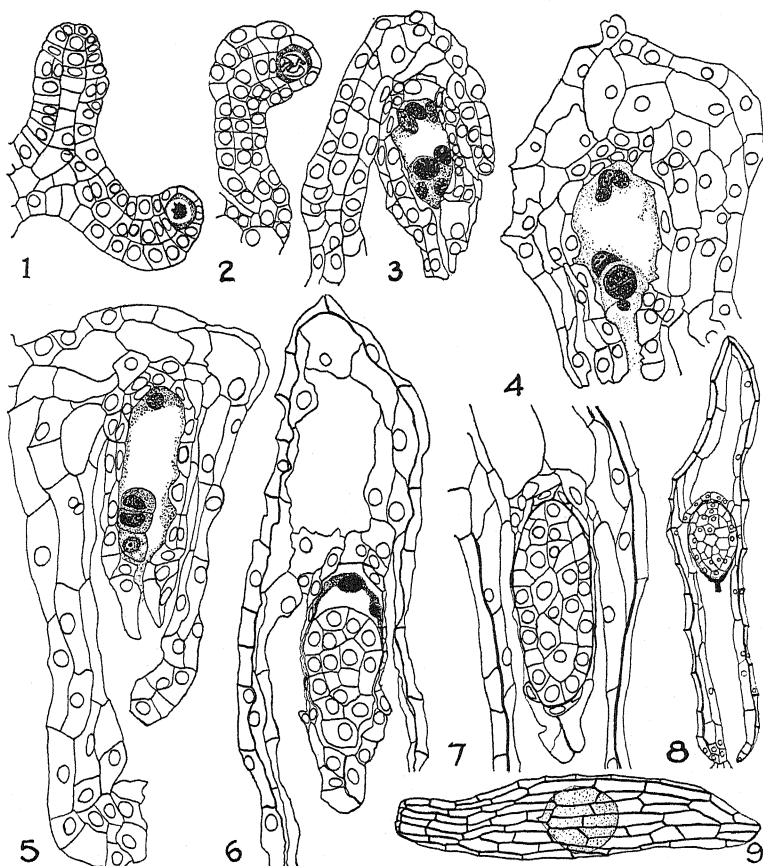
Young flower buds of *Cypripedium parviflorum* were covered with cheesecloth bags until the flowers had opened and their stigmas were receptive. The flowers were then cross-pollinated and rebagged. Pollinations were made on May 24, 28, and on June 5, 1936. Ovaries were fixed in Flemming's and Navashin's solutions just before pollination, and 2, 4, 5, 12, 14, 16, 26, 33, 55, and 72 days after pollination. The material was imbedded in paraffin; sections were cut 10 to 12  $\mu$  in thickness and stained in safranin and light green.

## Observations

### OVULE AND INTEGUMENTS

The ovary has a single cavity, with three parietal, forked placentae. In the ovaries of the earliest collections the ovules appear as small, hemispherical, or slightly oval protrusions, formed by rapidly dividing cells of the surface of the placentae. Each ovule primordium consists of a peripheral layer inclosing a single axial row of cells. The outermost cell of the axial row, the archesporial cell, enlarges and functions as the spore mother cell. The ovules increase in length and, in ovaries fixed 2 days after pollination, appear as in figure 1. At this time the spore mother cells have increased in size and have entered the early prophase stages of reduction division, and the first periclinal divisions leading to the formation of the inner integuments have occurred. By the fourth or fifth day following pollination (fig. 2) the stalks of the ovules have lengthened, chiefly by cell divisions, and the tips of the ovules have bent slightly to one side. The lobes of the inner integument are further advanced and the nuclei of the spore mother cells are in synapsis or late prophase stages of reduction division.

The collection of June 17 shows the funiculi of the ovules still longer, the tip of the ovule still more bent, the inner integument grown until it reaches the tip of the ovule, and most of the spore mother cells in metaphase or anaphase of reduction division. By this time the pollen tubes have reached the cavity of the ovary, having grown downward from the style along the inner surface of the ovary wall on either side of the base of each placenta. On June 21, 16 day after pollination, the ovules appear as in figure 3. The lobe of the inner integument has grown around the nucellus, forming the micropyle, and the lobe of the outer integument has begun to lengthen. The internal cells of the chalazal part of the ovule have begun to enlarge. Megasporogenesis is completed and the megagametophyte has enlarged and matured. The megagametophyte will be seen to contain seven, or possibly eight, nuclei. PACE (9) reports that, in *Cypripedium parviflorum*, *C. pubescens*, *C. candidum*, and *C. spectabile* (*C. hirsutum* Mill.), the megaspore mother cell divides once and the chalazal daughter cell enlarges and becomes the megagametophyte. The mature megagametophyte consists of four nuclei, two synergids and an egg at the micropylar end, and the fourth nucleus in the center or at the antipodal end. Although I have made no exhaustive study of megagametophyte development, it has been noticed that many of them in my preparations contained more than four nuclei. RUTGERS (12), SCHNARF (15), and MAHESHWARI (7) criticize PACE's work and list *Cypripedium* as showing a doubtful type of megagametophyte development. In view of these criticisms, I shall study the megagametophytes further and report the findings at a later time.



FIGS. 1-9.—Fig. 1, two ovules 2 days after pollination; upper one showing first divisions in formation of inner integument, lower showing spore mother cell in synapsis. Fig. 2, ovule 5 days after pollination; tip beginning to bend. Fig. 3, mature ovule 26 days after pollination, showing inner integument, micropyle, beginning of outer integument, and mature megagametophyte. Fig. 4, young seed 33 days after pollination and shortly after fertilization; 2-celled embryo, remains of megagametophyte; inner cells of chalaza and funiculus beginning to vacuolate and enlarge. Fig. 5, seed 33 days after pollination, young embryo with suspensor; funiculus and outer integument elongating. Fig. 6, chalazal portion of seed 72 days after pollination, showing chalazal cavity, remains of megagametophyte, nucellus disintegrated, inner integument shriveled, inner and side walls of cells of outer layer of funiculus and outer integument thickened, inner cells of funiculus and outer integument disintegrating, embryo enlarging. Fig. 7, central portion of seed 72 days after pollination, showing larger embryo and its relation to cells of seed coat; inner integument still more shriveled. Fig. 8, longitudinal section of entire seed; seed coat consisting of funiculus and outer integument; shriveled inner integument forming envelope around embryo; embryo suspended at apical end. Fig. 9, ripe seed; transparent coat consisting of single layer of cells; embryo suspended in pocket of air.

## EMBRYO AND SEED COAT

During the season of 1936, when the material for this study was collected, fertilization occurred early in July, between 26 and 33 days after pollination. Within a week after fertilization, the zygote had divided by a wall transverse to the long axis of the megagametophyte (fig. 4). The daughter cell on the micropylar side of the embryo does not divide again, but becomes the single-celled suspensor. The other daughter cell usually divides by a wall parallel to the first (fig. 5), but sometimes by a wall perpendicular to it. Division continues in three planes, and by August 16, in ovaries pollinated on June 5, the embryo consists of an oval mass of undifferentiated meristematic cells, and a single-celled suspensor (fig. 6), which eventually disappears (fig. 7). No endosperm is produced.

While the embryo is growing, the whole ovule undergoes rapid enlargement, due primarily to the intake of water and the vacuolation of the cells. Early indications of this are seen in figure 3, where the inner cells in the chalazal region are somewhat enlarged. Further stages in this enlargement are seen in figures 4 and 5. The cells of the outer integument divide and elongate so that the outer integument grows beyond the tip of the inner integument. The funiculus also elongates and pushes the young seed out into the cavity of the capsule (figs. 5, 6). The nucellus begins to degenerate. Eventually the enlarged inner cells of the chalaza separate and disintegrate, producing the chalazal cavity (figs. 6, 8). Those cells of the chalaza in the vicinity of the apical part of the embryo are not disrupted, however, and they form a bridge suspending the embryo, with the remains of the surrounding nucellus and inner integument, in the cavity of the seed (figs. 6-8).

When the embryo has reached a many-celled stage, the nucellus has disappeared and the inner integument has begun to shrivel and degenerate. The funiculus, chalazal region, and outer integument take part in the formation of the seed coat. The internal cells of these parts gradually disintegrate and leave the external single layer of cells as the seed coat. The inner and end walls of cells of the seed coat thicken, while the outer walls remain thin. The contents of the cells of the seed coat usually disappear.

By the middle of August, ovaries contain seeds in different stages of maturity, but most of them appear as in figure 8. The embryo is still a spherical or oval mass of undifferentiated cells. It is surrounded by an envelope which consists of the shriveled and disorganized remains of the inner integument. The embryo, with its envelope, is suspended in a cavity within the seed coat by bands of living cells at its apical end. The seed coat still consists of the two outer layers of cells of the funiculus, chalaza and outer integument, but the inner layer shows evidence of disorganization.

No vascular tissue is present in the funiculus, and all food and water must therefore pass from cell to cell through the funiculus and the suspending bridge

of living cells to the embryo. The parenchymatous cells of the ovary wall and placenta are filled with starch grains throughout the summer. Starch appears early in the inner parenchymatous cells of the ovule and disappears only when they disintegrate, toward the close of the period of seed maturation. No starch is found in the embryo.

#### MATURE SEED

Capsules usually begin to dry and turn brown and seeds begin to ripen in early September, in Evanston. A mature seed is shown in figure 9. The seed coat is now a single-layered, transparent sac, open at the end which breaks from the placenta and closed at the opposite end. All the cells formerly inside this layer have disintegrated. The embryo is surrounded by the shriveled inner integument, which can be removed by dissection. The embryo, with its envelope, is suspended in the center of the sac by a few strands of cells which can be seen adhering to the embryo when it is removed from the seed coat. The embryo is bounded by an epidermal layer which is only slightly differentiated. In some embryos the cells at the apical, or bud, end are smaller and more densely filled with cytoplasm than are those at the opposite end.

Microchemical tests were made to determine the composition of walls and inclusions of cells of the mature seed. The methods used are described in ECKERSON's unpublished manual of microchemistry. The thickened inner and side walls of the cells of the seed coat give a positive test for lignin with phloroglucin and hydrochloric acid, and the thin outer walls give a positive test for cellulose with iodine and sulphuric acid. The lignin and cellulose are deposited unevenly on the walls, in strips or bars. This is especially evident on those cells near the open end of the seed. None of the walls of cells of the seed coat gives a positive test for cutin with Sudan III. NETOLITZKY (8) states that the inner and side walls of the cells of the seed coat are cutinized. The cells of the seed coat may be empty or may contain granules which stain yellow-brown with iodine. The walls of cells which suspend the embryo and those of the embryo itself give positive tests for cellulose. The covering of the embryo gives negative tests for lignin, cellulose, and cutin, with the methods used. The cells of the embryo, especially the large cells at the posterior end, contain droplets of oil. Starch, sugar, and nitrates are absent from the mature seed coat and embryo.

The seeds of most orchids are very light and buoyant because of their small size and their saclike, air-filled coats. They are readily carried by the wind and they float on water. The loss of seeds in nature by wind or by run-off water is probably great.

Nothing is known as to how the embryo obtains water for germination. The air inside the coat is not easily removed or replaced by water—not even when suction is applied. Since the embryo is suspended from the coat in a pocket of air,

it must obtain water for germination by imbibition through the coat and along the strands of cells by which it is suspended, or from the air itself. Since the cell walls of the coat are not cutinized, such imbibition may occur. Of course, if the coat decays, water could reach the embryo, but no information on this point was obtained.

### Summary

1. The anatropous ovule is formed in the usual manner from the placenta. The enlarged archesporial cell becomes the spore mother cell, which divides by meiosis.

2. The mature megagametophyte has been reported to contain four nuclei, but in my material it seems to be of the usual 8-nucleate type. More evidence is required to settle this point.

3. The zygote divides transversely to the long axis of the megagametophyte. The micropylar daughter cell becomes a one-celled suspensor and the chalazal daughter cell becomes the embryo proper. The wall dividing the chalazal daughter cell is usually parallel to the first, but may be at right angles to it. The mature embryo is a spherical, or spheroidal, mass of undifferentiated—or only slightly differentiated—cells which have cellulose walls and which contain droplets of oil, but no starch or sugar.

4. The nucellus disintegrates at an early stage of seed development. The inner integument shrivels and becomes a skinlike covering around the embryo. No endosperm is produced.

5. The seed coat is formed from the funiculus, chalaza, and outer integument, the inner cells of which disintegrate, leaving only the outer layer. The inner and side walls of the cells of the seed coat are composed of lignin and the outer walls of cellulose. No cutin was found on the seed coat.

6. A cavity is formed at the chalazal end of the seed by rupture and disintegration of inner cells and at the micropylar end by growth of the funiculus and outer integument. The embryo is suspended in this air-filled cavity by certain cells of the chalazal region.

7. The air inside the seed coat makes the seeds buoyant and may make it difficult for the embryo to obtain water for germination.

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EFFECT OF LIGHT AND TEMPERATURE ON FLORAL  
INITIATION IN COCKLEBUR AND  
BILOXI SOYBEAN<sup>1</sup>

CONTRIBUTIONS FROM THE HULL BOTANICAL LABORATORY 516

WILLIAM E. SNYDER

(WITH FIVE FIGURES)

Introduction

In recent work with Biloxi soybean (*Soja max*) and cocklebur (*Xanthium pennsylvanicum*), HAMNER (6) has shown that photoperiodic induction takes place as a result of reactions which occur during both the light and the dark period of a photoinductive cycle. Disregarding the results dealing with light intensity and temperature, the following scheme may illustrate the response on the part of the plant to given treatments. For purposes of simplicity, the only variations introduced are those of duration of the photoperiods and the dark periods during particular treatments with cyclic alternations of light and darkness.

1. If the light period is of a given duration (about 12 hours) and the dark period is also of a given duration, the plants will flower as a result of treatment.
2. If the dark period is shorter than 8 hours, the plants will fail to flower, regardless of the duration of the photoperiod.
3. If the photoperiod is very short, even though the dark period is relatively long, the plants may fail to flower; the response depends upon the previous conditions to which the plant has been exposed.
4. If both the photoperiods and the dark periods are relatively short, the plants will fail to flower, and such treatment serves to inhibit flowering following subsequent photoperiodic cycles which might otherwise prove effective in bringing about induction.

As a result of these observations, HAMNER has presented the following relationship with regard to Biloxi soybean and cocklebur. If A represents the changes or conditions related to exposure to light, and B represents those related to exposure to darkness, then A, B→C represents the interrelationship between A and B which brings about initiation of floral primordia, the latter condition being designated by C.

This interaction takes place in condition (1) above. In condition (2), the reac-

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tions leading to floral initiation do not take place because of limitation in the effectiveness of B. Under condition (3), floral initiation does not take place because of limitation in the effectiveness of A. Under condition (4), the reactions are limited by the ineffectiveness of both A and B. In this last case exposure to very short cycles of alternating light and darkness results in decreased effectiveness of both A and B, regardless of the previous treatments; subsequent to such cycles, conditions must be attained which will result in an increase in the effectiveness of both A and B in order that floral initiation may result.

Thus the cocklebur plants growing on a long photoperiod in the greenhouse remain vegetative under condition (2). Such plants may reach a high degree of effectiveness of A, and are induced to flower by a single treatment of a long dark period, since their flowering is limited only by the effectiveness of B. If such plants are removed from the greenhouse and treated with a number of short cycles of alternating light and darkness, however, the effectiveness of A decreases. Subsequently such plants must be exposed to a photoinductive cycle, which includes both a relatively long light period and a relatively long dark period.

The present work deals with these various interrelationships and the influence of temperature, light intensity and duration, and other environmental factors upon these interactions.

#### General methods

Two short-day plants, cocklebur and Biloxi soybean, were employed. The experiments with cocklebur were performed during the summer and early autumn of 1939, while the experiments with soybean were conducted during the spring and summer of 1940.

The cockleburs were grown from seed collected in the vicinity of Chicago in November, 1938. The burs were stored out-of-doors until desired for use. The seeds were removed from the fruit, scratched slightly to rupture the seed coats, and planted within a few days in light potting soil. Before germination of the seeds, the flats were placed on a well-lighted bench in the greenhouse, where supplementary illumination of about 100 foot-candles at the soil surface was provided by Mazda filament light from sundown until 2:00 A.M., making a photoperiod never less than 18 hours. The conditions on this bench will subsequently be referred to as those of long photoperiod. Cocklebur and other short-day plants have been grown under these conditions for more than a year without showing evidence of floral initiation when dissected. When the seedlings were about 4 inches tall they were transplanted to  $3\frac{1}{2}$ -inch clay pots.

Four seeds of Biloxi soybean were planted in  $4\frac{1}{2}$ -inch clay pots containing garden soil and placed immediately on long photoperiod. After the seedlings had emerged and the cotyledons were well expanded, two were removed from each pot, care being taken to secure maximum uniformity of plants.

The plants were watered twice daily with tapwater and were frequently given a double-strength modified SHIVE'S (12) solution. They were exposed to conditions of long photoperiod until treated. The cockleburs were 6-8 weeks old and the soybeans 4-5 weeks old when the experiments were started. At the end of a specific treatment, if the intensity of sunlight in the greenhouse was 1000 foot-candles or more, the plants were placed immediately on long photoperiod; if less than this, the cockleburs were placed in a room at 70° F. illuminated with a carbon arc and the soybeans under white fluorescent light (1000 foot-candles or more) until the sunlight was sufficiently strong for them to be placed under conditions of long photoperiod. After treatment, all plants remained under conditions of long photoperiod for 3 weeks, at which time dissections were made. At the start of each experiment, and from time to time during them, plants which had been continuously under conditions of long photoperiod were dissected to make certain that no floral initiation had taken place. Illumination was supplied by sunlight, Mazda filament light, carbon arc light, or white fluorescent light. Intensities were determined in foot-candles by means of a standardized Weston photometer.

In experiments 1 and 2 with cocklebur, and in experiments 8-10 with soybean, the plants were treated with short cycles of alternating light and darkness, each consisting of 3 hours of darkness followed by 3 minutes of light. In the remaining experiments with cocklebur (experiments 3-7) these short cycles consisted of 2 hours of darkness followed by 2 minutes of light. The light intensity of these short photoperiods was 200 foot-candles unless otherwise indicated.

### Procedure and results

#### Cocklebur

The experiments were of two general types: one dealt with the effectiveness of short alternating periods of darkness and light under various conditions in preventing floral initiation, and the other with the effects of light and temperature—both separate and in combination—during the photoperiod on floral initiation and development.

#### A. EFFECTIVENESS OF SHORT ALTERNATING PERIODS OF DARKNESS AND LIGHT UNDER VARIOUS CONDITIONS IN PREVENTING FLORAL INITIATION

It has been shown that vegetative cockleburs kept constantly on long photoperiod may fail to be induced if just subsequent to a long photoperiod and prior to the 15-hour dark period a series of relatively short cycles of alternating light and dark periods are given (6). The same procedure was employed here in the first of these experiments, but in all subsequent ones it was slightly modified.

EXPERIMENT 1.—The first experiment was begun June 29, 1939. One hundred and sixty plants growing on long photoperiod were selected for uniformity. Ten of

these, designated continuous long photoperiod controls, were left on conditions of long photoperiod. Twenty were placed in a darkroom immediately; of these, ten were given a 15-hour and ten a 36-hour continuous dark period and then returned to conditions of long photoperiod. One group of ten plants was given a single short cycle of 3 hours of darkness and 3 minutes of light, subjected to a 15-hour dark period, and then returned to conditions of long photoperiod; another lot was given two of these short cycles, followed by a 15-hour dark period, and returned to long photoperiod; and so on. The maximum number of short cycles which any lot received was twelve. The remaining ten plants were given twelve of these cycles and returned immediately to long photoperiod without being exposed to a 15-hour dark period. The results (table 1) indicate that the cyclic treatment,

TABLE 1

EFFECT OF NUMBER OF SHORT CYCLES, EACH CONSISTING OF 3 HOURS OF DARKNESS AND 3 MINUTES OF LIGHT, ON FLORAL INITIATION IN COCKLEBUR WHEN PLANTS RECEIVED A LONG DARK PERIOD IMMEDIATELY SUBSEQUENT TO TREATMENT WITH SHORT CYCLES. TEN PLANTS IN EACH LOT

No. of short cycles. . .	0	0	0	1	2	3	4-12
Length of dark period following short cycles (hours) . . . . .	0	15	36	15	15	15	15
Flowering condition after 14 days on long photoperiod. . . . .	10 veg.	10 fl. pr.	10 fl. pr.	4 fl. pr., 6 veg.	10 veg.	10 veg.	10 veg.

following a long photoperiod, may prevent the effectiveness of a subsequent 15-hour dark period in initiating floral primordia.

EXPERIMENT 2.—On the basis of the results of experiment 1, a second was carried out in August, 1939. Three relatively short cycles of 3-hour dark periods and 3-minute light periods, shown by the preceding results to be sufficient to prevent initiation, were used. Upon dissection, however, it was found that many of the controls (which received a 15-hour dark period following the three cycles) had initiated floral primordia.

June 29 was intermittently cloudy, and the light intensity was therefore rather low. Experiment 1 was begun at 10:00 A.M. on that day, so these plants had been subjected to approximately 5 hours of low-intensity light. Experiment 2 was started at 5:00 P.M. following an exceptionally bright day. Since this difference might have had an important effect on the number of relatively short alternating light and dark periods required to inhibit floral initiation, a thorough investigation was made of the effects of the environmental factors on the effectiveness of the cyclic treatment.

For uniformity, all remaining experiments (3-10) were begun at 5:00 P.M. fol-

lowing at least 10 hours of sunlight of 3000-5000 foot-candles' intensity. Experiments 3-7 were carried out during the first week of September, and dissections were made during the first week of October.

EXPERIMENT 3.—Three groups of vegetative plants, each consisting of 168 plants, were exposed continuously to light for 36 hours, one group at an intensity of 50 foot-candles, another at 500, and another at 5000. From each of these groups sixteen plants were placed in the darkroom. Of each group of sixteen, half received a 12-hour and half a 36-hour continuous dark period and then returned to long photoperiod. From each of the original groups, 152 plants were given short cycles of 2 hours of darkness followed by 2 minutes of light of 200 foot-

TABLE 2

EFFECT ON FLORAL INITIATION IN COCKLEBUR OF VARIOUS LIGHT INTENSITIES DURING 36-HOUR PHOTOPERIOD BEFORE CYCLIC TREATMENT CONSISTING OF 2 HOURS OF DARKNESS AND 2 MINUTES OF LIGHT. DATA TAKEN 21 DAYS AFTER COMPLETION OF TREATMENT. EACH LOT OF EIGHT PLANTS

No. of short cycles.....	0	0	0	1	2	3	4	5-18	18
Length of dark period following short cycles (hours)...	0	12	36	12	12	12	12	12	0
Intensity of 36-hour photo-period:									
50 f.c.....	8 veg.	8 veg.	8 veg.	8 veg.	8 veg.	8 veg.	8 veg.	8 veg.	8 veg.
500 f.c.....	8 veg.	8 fl.	8 fl.	8 fl.	5 fl., 3 veg.	8 veg.	8 veg.	8 veg.	8 veg.
5000 f.c.....	8 veg.	8 fl.	8 fl.	8 fl.	8 fl.	6 fl., 2 veg.	4 fl., 4 veg.	8 veg.	8 veg.

candles at 70° F. Lots of eight plants were then removed from each group at the end of every cycle and subjected to a 12-hour continuous dark period before being returned to long photoperiod. One set of plants, however, which had received each of the light intensities prior to the cyclic treatment, was returned directly to long photoperiod after eighteen cycles, no 12-hour dark period having preceded this return. Eight plants, selected for controls for experiments 3-7 and left on long photoperiod, were vegetative at the conclusion of the experiment.

The results (table 2) indicate the reason for the variation observed between experiments 1 and 2. Apparently the degree of effectiveness of the cyclic treatment is directly related to the intensity and duration of light which the plants receive just prior to the short cycles of darkness and light; that is, plants exposed to high-light intensity preceding the cyclic treatment require more of these short cycles to prevent subsequent floral initiation than do those exposed to a relatively low-light intensity prior to the cyclic treatment.

Since these short cycles were effective in inhibiting subsequent floral initiation in the treatments, the effect of continuous photoperiod of low light intensity, as compared with these short cycles, was next determined. An attempt was made also to determine the effect of variation in the light intensity during the short photoperiods of these short cycles.

EXPERIMENT 4.—Four hundred and eighty-eight cocklebur plants, growing on long photoperiod, were selected for uniformity. Sixteen of these were placed in the darkroom, eight being returned to long photoperiod after a 12-hour and

TABLE 3

EFFECT ON FLORAL INITIATION IN COCKLEBUR OF CYCLIC TREATMENT CONSISTING OF 2 HOURS OF DARKNESS AND 2 MINUTES OF LIGHT OF VARIOUS INTENSITIES AND OF CONTINUOUS LIGHT OF LOW INTENSITY. EACH LOT OF EIGHT PLANTS

No. of cycles.....	1	2	3	4	5	6	7	8	9-18	18
No. of hours of continuous darkness following cyclic treatment.....	12	12	12	12	12	12	12	12	12	0
Flowering condition after 21 days on long photoperiod, when cycle consisted of:										
2 hours of darkness plus 2 minutes of light at:										
2000 f.c.....	8 fl.	8 fl.	5 veg., 3 fl.	4 veg., 4 fl.	7 veg., 1 fl.	8 veg.	8 veg.	8 veg.	8 veg.	8 veg.
200 f.c.....	8 fl.	8 fl.	2 veg., 6 fl.	4 veg., 4 fl.	8 veg.	8 veg.	8 veg.	8 veg.	8 veg.	8 veg.
20 f.c.....	8 fl.	8 fl.	3 veg., 5 fl.	5 veg., 3 fl.	8 veg.	8 veg.	8 veg.	8 veg.	8 veg.	8 veg.
2 hours of continuous light at 20 f.c.	8 fl.	8 fl.	8 fl.	8 fl.	8 fl.	8 fl.	8 fl.	7 veg., 1 fl.	8 veg.	8 veg.

eight after a 36-hour continuous dark period. These control plants initiated primordia. One hundred and twelve plants were placed under continuous light of 20 foot-candles. Every 2 hours for the first 20 and every 4 hours for the next 16, lots of eight plants each were removed to the darkroom for 12 hours and then immediately returned to long photoperiod. Three lots of 120 plants each were given short cycles with the 2-minute light periods at intensities of 20, 200, and 2000 foot-candles, respectively. Each of the eighteen short cycles consisted of 2 hours of darkness followed by 2 minutes of light. Lots of eight plants were removed from each intensity at the end of every cycle, given a 12-hour continuous dark period, and returned to long photoperiod. At the completion of eighteen cycles an additional group of eight plants from each intensity was returned directly to long photoperiod with no intervening dark period.

The results (table 3) show that for cocklebur, cycles with brief periods of light of all three intensities are effective in inhibiting floral initiation, even though the plants are given a subsequent 12-hour dark period. Those plants given cycles with 2-minute light periods at 2000 foot-candles required seven cycles for such inhibition, while those given 20 or 200 foot-candles required only five cycles. The plants given 18 hours or more of continuous light of low intensity (20 foot-candles) also failed to initiate floral primordia, even when subsequently given a 12-hour dark period.

Table 3 indicates that light intensity during the treatment of short cycles may influence the results. In the following experiment an attempt was made to determine what effect temperature might have on treatment with short cycles.

TABLE 4

EFFECT ON FLORAL INITIATION IN COCKLEBUR OF VARIOUS TEMPERATURES DURING SHORT CYCLES CONSISTING OF 2-HOUR DARK AND 2-MINUTE LIGHT PERIODS. DATA TAKEN 21 DAYS AFTER COMPLETION OF TREATMENT. EACH LOT OF EIGHT PLANTS

No. of short cycles....	1	2	3	4	5	6-18	18
Length of dark period following short cycles (hours).....	12	12	12	12	12	12	0
Flowering condition after 21 days on long photoperiod, following short cycles at:							
40° F.....	8 fl.	8 fl.	1 veg., 7 fl.	2 veg., 6 fl.	7 veg., 1 fl.	8 veg.	8 veg.
75° F.....	8 fl.	8 fl.	2 veg., 6 fl.	4 veg., 4 fl.	8 veg.	8 veg.	8 veg.
95° F.....	8 fl.	8 fl.	1 veg., 7 fl.	3 veg., 5 fl.	8 veg.	8 veg.	8 veg.

EXPERIMENT 5.—The continuous long photoperiod controls of this experiment are those of experiment 3, and the 12- and 36-hour continuous dark-period controls (not given the short-cycle treatment) are those of experiment 4. Three hundred and sixty plants were divided into three lots of 120 plants each and subjected to the short-cycle treatment at 40°, 75°, and 95° F., respectively. Lots of eight plants were removed from each group at the end of every short cycle, given a 12-hour continuous dark period at 70° F., and returned to long photoperiod. The results of this experiment are given in table 4. The differences in response to the three temperature groups are insignificant and indicated that the temperatures used were without influence on the effectiveness of the alternating dark and light periods in preventing floral initiation.

#### B. DURATION AND INTENSITY OF LIGHT AND TEMPERATURE REQUIREMENTS OF PHOTOPERIOD PRECEDING DARK PERIOD

Since it has been observed that floral initiation could be prevented by treating plants with short cycles of alternating darkness and light before subjecting them

to a continuous dark period of 9 hours or more, it was decided to test the conditions under which plants might resume a condition permitting initiation by giving some which had received eighteen short cycles a controlled photoperiod before subjecting them to the continuous dark period of at least 12 hours.

EXPERIMENT 6.—Eighty vegetative cocklebur plants were selected from long photoperiod and given eighteen cycles of alternating 2-hour dark periods and 2-minute light periods (200 foot-candles) at 75° F. Ten plants were returned to long photoperiod immediately after the eighteen cycles, and served as checks. The remaining seventy were divided equally into seven groups, each of which was given one of the following treatments:

- 2 hours of 2000 f.c. carbon arc light + 22 hours darkness
- 4 hours of 2000 f.c. carbon arc light + 20 hours darkness
- 6 hours of 2000 f.c. carbon arc light + 18 hours darkness
- 8 hours of 2000 f.c. carbon arc light + 16 hours darkness
- 10 hours of 2000 f.c. carbon arc light + 14 hours darkness
- 10 hours of 100 f.c. Mazda filament light + 14 hours darkness
- 10 hours of 10 f.c. Mazda filament light + 14 hours darkness

Each cycle was repeated three times, at the conclusion of which the plants were returned immediately to long photoperiod, where they remained for 21 days.

TABLE 5

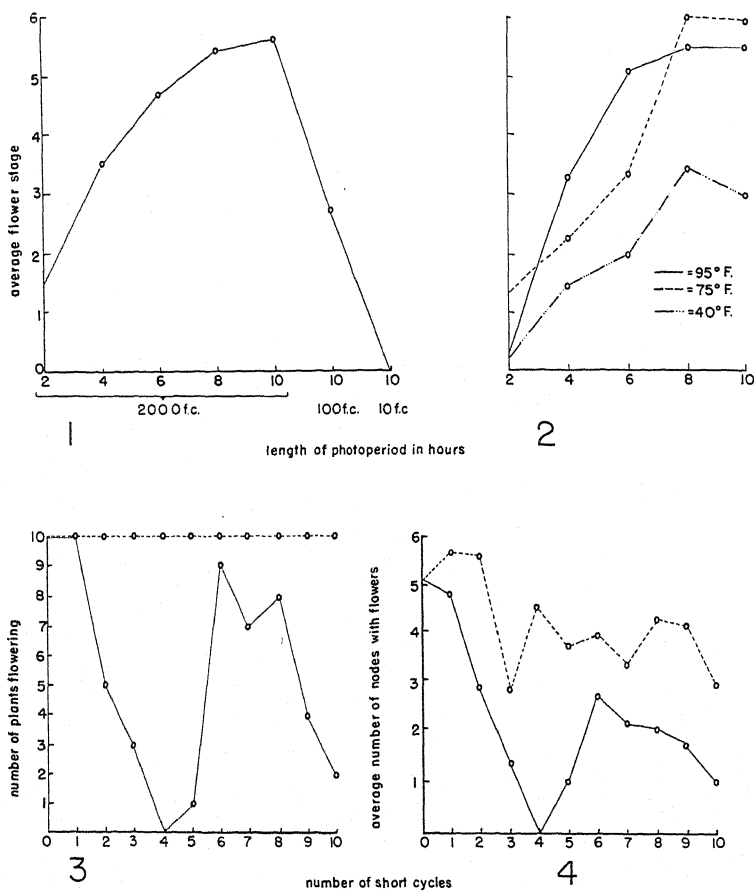
FLOWERING RESPONSE OF COCKLEBUR TO VARIOUS PHOTOINDUCTIVE CYCLES GIVEN AFTER 18 CYCLES OF ALTERNATING 2-HOUR DARK AND 2-MINUTE LIGHT PERIODS. EACH PHOTOINDUCTIVE CYCLE REPEATED THREE TIMES. DATA TAKEN 21 DAYS AFTER COMPLETION OF TREATMENT. EACH LOT OF TEN PLANTS

PHOTOINDUCTIVE TREATMENT	No. OF PLANTS	
	VEG.	FL. PR.
2 hours of 2000 f.c.+22 hours of darkness.....	2	8
4 hours of 2000 f.c.+20 hours of darkness.....	0	10
6 hours of 2000 f.c.+18 hours of darkness.....	0	10
8 hours of 2000 f.c.+16 hours of darkness.....	0	10
10 hours of 2000 f.c.+14 hours of darkness.....	0	10
10 hours of 100 f.c.+14 hours of darkness.....	0	10
10 hours of 10 f.c.+14 hours of darkness.....	10	0
Controls (continuously on long day).....	10	0

In two groups, the controls and those given three cycles of 10 foot-candles Mazda filament light for 10 hours, there was no initiation of floral primordia (table 5). Two of the plants exposed to three cycles of 2000 foot-candles of carbon arc light for 2 hours and 22 hours of darkness also failed to initiate primordia. The apical meristems had become flower primordia in all other plants. The stages of



flower development were arbitrarily classified, and an estimate as to the effectiveness of the different treatments was obtained. Stage 1 represents the first indica-



FIGS. 1-4.—Fig. 1, following eighteen cycles of alternating short dark and light periods, effect of light intensity and duration of photoperiod of 24-hour photoinductive cycle on floral development in cocklebur. Each cycle repeated three times. Data taken 21 days after completion of treatment. Fig. 2, effect on floral development of temperature during photoperiods of various durations, following eighteen cycles of alternating short dark and light periods. Each photoperiod 2000 foot-candles, followed by 14-hour dark period at 70° F. Each cycle repeated three times. Data taken 21 days after completion of treatment. Figs. 3, 4, effect on initiation in Biloxi soybean of ten photoinductive cycles which include short cycles consisting of 3 hours of darkness and 3 minutes of light (200 f.c.). Short cycles preceding dark period represented by broken line; those following, by solid line. Data taken 21 days after completion of treatment.

tion of initiation; stage 6, macroscopic flowers; the other stages range between. Figure 1 represents the average stage of flowering of all plants in a group and shows

that the longer the duration and the stronger the intensity of the photoperiod, the more rapid the subsequent rate of floral development.

EXPERIMENT 7.—One group of workers (4, 5, 10, 11, 13, 14) has reported that variations in temperature during the photoperiod may alter the length of the light period required for floral initiation in many plants. Others (4, 5, 7, 9, 10) have shown that the length or number of critical dark periods may be adversely affected by temperature conditions during the dark period.

Experiment 7 shows that the temperature conditions prevailing during the photoperiod affect initiation and subsequent flower development. It varies from ex-

TABLE 6

FLOWERING RESPONSE OF COCKLEBUR TO VARIATIONS IN TEMPERATURE DURING PHOTOPERIOD OF VARIOUS PHOTOINDUCTIVE CYCLES FOLLOWING 18 CYCLES OF ALTERNATING 2-HOUR DARK AND 2-MINUTE LIGHT PERIODS. TEMPERATURE OF DARK PORTION OF PHOTOINDUCTIVE CYCLE 70° F. DATA TAKEN 21 DAYS AFTER COMPLETION OF TREATMENT. EACH LOT OF EIGHT PLANTS

	TEMPERATURE DURING PHOTOPERIOD					
	40° F.		75° F.		95° F.	
	VEG.	FL.	VEG.	FL.	VEG.	FL.
After 18 cycles of alternating light and dark periods, subjected to three photoinductive cycles:						
2 hours of 2000 f.c.+14 hours dark. ....	7	1	1	7	7	1
4 hours of 2000 f.c.+14 hours dark. ....	1	7	0	8	0	8
6-10 hours of 2000 f.c.+14 hours dark. ....	0	8	0	8	0	8
Controls (continuously on long photoperiod).....	8 vegetative, 0 flowering					
After 18 cycles transferred directly to long photoperiod. ....	8 vegetative, 0 flowering					
After 18 cycles transferred to 12-hour dark period, then to long photoperiod. ....	8 vegetative, 0 flowering					
Transferred to 14-hour dark period immediately after photoperiod, then to long photoperiod. ....	0 vegetative, 8 flowering					

periment 6 in two respects: (a) all plants were given 12-hour dark periods, and (b) three temperature conditions prevailed during the photoperiod. The results (table 6) show that initiation was inhibited at low and high temperatures, but only when the treatment consisted of three cycles of 2 hours of 2000 foot-candles of carbon arc light and 12-hour dark periods. Significant variations were found in the rate of development of the flower primordia (fig. 2). In general, at the higher temperatures there was greater increase in the rate of flower-bud development than at the low temperature. At all three temperatures a marked increase in the rate of flower-bud development followed three photoinductive cycles whose photoperiods

were 8 hours long. Plants given three cycles with 10-hour photoperiods had not developed so far as those given an 8-hour photoperiod.

### Biloxi soybean

Relatively short alternating dark and light periods were inserted into the photoinductive cycle of another short-day plant to test the effectiveness of the cyclic treatment in preventing floral initiation. Soybean was selected because of the data available relative to its photoperiodic relationships. As in the case of cocklebur, short cycles were given between the photoperiod and the dark period of each photoinductive cycle, and similar short cycles were inserted between each photoinductive cycle (following the dark period of each photoinductive cycle).

EXPERIMENT 8.—On May 3, 1940, 250 soybeans which had been on long photoperiods for 5 weeks were selected. Five lots of ten plants each were used as controls and given one of the following treatments:

1. Left continuously on long photoperiod.
2. Given 240 hours of white fluorescent light of 600 foot-candles, then returned to long photoperiod.
3. Given 240 hours of continuous darkness, then returned to long photoperiod.
4. Given 80 short cycles (240 hours) consisting of 3 hours of darkness and 3 minutes of light (200 foot-candles Mazda filament light), then returned to long photoperiod.
5. Given ten photoinductive cycles consisting of 10 hours of light followed by 14 hours of darkness, then returned to long photoperiod.

All plants of lots 1-4 were vegetative, but those of lot 5 were flowering at the conclusion of the experiment. The remaining 200 plants were divided into two equal lots, and at 5:00 P.M., following a photoperiod of more than 10 hours of sunlight of 3000-5000 foot-candles, one (group A) was placed in the darkroom, while the other (group B) was placed in a darkroom equipped to give frequent short periods of Mazda filament light.

Following a 14-hour continuous dark period, the 100 plants of group A were removed to the short-cycle room. One lot of ten plants received one short cycle consisting of 3 hours of darkness followed by 3 minutes of light, then placed under white fluorescent light (600 foot-candles) for 10 hours, and finally returned to the darkroom for 14 hours. Another lot received two of these short cycles following the 14-hour dark period; a third received three of these cycles, etc. Each of these treatments (10 hours of light plus 14 hours of darkness plus 1-10 short cycles) was repeated ten times. At the conclusion of a given treatment the plants were returned to conditions of long photoperiod in the greenhouse—provided the intensity of the sunlight exceeded 1000 foot-candles—or were placed under white fluorescent light until such time as the intensity of the sunlight was 1000 foot-candles or more.

Further, any group which completed the treatment during the late afternoon was kept under the fluorescent light until the following morning.

Of the 100 plants of group B, placed in the short-cycle room at the start of the experiment, one lot of ten plants remained for one short cycle, another for two, and so on. Upon removal from this room, the plants were given a 14-hour continuous dark period, followed by 10 hours of white fluorescent light, and then returned to the short-cycle room for the specified number of short cycles. Each of these treatments was likewise repeated ten times, and at the conclusion of a given treatment the plants were returned to conditions of long photoperiod.

The results (fig. 3) are striking, in that short cycles inserted before the dark period were ineffective in preventing floral initiation, while the inhibitory effect of short cycles following the dark period appears rhythmical. Although all plants were flowering when the short cycles preceded the dark period, some inhibitory effect on flowering is evidenced by the average number of nodes with flowers per plant. Figure 4 shows that the same general trends of inhibition of the number of nodes with flowers is affected by short cycles inserted either before or after the dark period, as well as by the effect of short cycles inserted after the dark period on the total number of plants flowering. A comparison of the average number of nodes per plant with flower primordia of comparable treatments from groups A and B shows that in no instance was there a greater number of nodes with primordia when the short cycles followed the dark period than when they preceded it. Evidently short cycles inserted after the dark period of the photoinductive cycle are much more effective in suppressing the flowering response.

EXPERIMENT 9.—This experiment was performed at the same time as experiment 8, hence the controls for both were the same. Seven hundred soybeans which had been on continuous long photoperiods were selected for uniformity and divided into seven groups of 100 plants each. These groups were subjected to cycles as follows:

1. 10 hours of light, then 14 hours of continuous darkness.
2. 10 hours of light followed by one short cycle (3 hours of darkness and 3 minutes of 200 foot-candles of Mazda filament light), then 14 hours of continuous darkness.
3. 10 hours of light followed by five short cycles, then 14 hours of continuous darkness.
4. 10 hours of light followed by ten short cycles, then 14 hours of continuous darkness.
5. 10 hours of light followed by 14 hours of continuous darkness, then one short cycle.
6. 10 hours of light followed by 14 hours of continuous darkness, then five short cycles.

7. 10 hours of light followed by 14 hours of continuous darkness, then ten short cycles.

Ten plants from each group were removed to conditions of long photoperiod at the end of each cycle, ten cycles being required to remove all plants from any group.

The effect of the short-cycle treatments, combined with a varying number of photoinductive cycles, upon the total number of plants flowering per treatment is given in table 7. In no instance were flowers induced when less than three photoinductive cycles were used; and as has been previously reported (1, 3, 9), maximum flowering occurred when a relatively large number of photoinductive cycles were given.

TABLE 7

EFFECT ON FLORAL INITIATION IN SOYBEAN OF VARYING NUMBER OF PHOTOINDUCTIVE CYCLES WHICH INCLUDE SHORT CYCLES CONSISTING OF 3 HOURS OF DARKNESS AND 3 MINUTES OF LIGHT (200 F.C.). DATA TAKEN 21 DAYS AFTER COMPLETION OF TREATMENT. EACH LOT OF TEN PLANTS

TREATMENT	NO. OF PLANTS FLOWERING AFTER NUMBER OF PHOTOINDUCTIVE CYCLES AS INDICATED									
	1	2	3	4	5	6	7	8	9	10
No. of short cycles inserted in photoinductive cycle before 14-hour dark period:										
1.....	0	0	2	2	8	8	9	10	10	10
5.....	0	0	0	0	0	3	0	3	8*	9*
10.....	0	0	0	0	0	2	1	6	8	10
No. of short cycles inserted in photoinductive cycle after 14-hour dark period:										
1.....	0	0	0	1	4	9	10	10	10	10
5.....	0	0	0	0	0	0	0	0	2	1
10.....	0	0	0	0	2	0	1	5	3	2
No short cycles included in photoinductive cycle.....	0	0	6	8	10	10	10	10	10	10
Continuous long photoperiod	0 flowering; 10 vegetative									

\* One dead plant in this lot.

One short cycle inserted either before or after the dark period of the photoinductive cycle had some effect when relatively few photoinductive cycles were given (seven or less), but when eight or more comprised the treatment all plants showed some degree of flowering. If five short cycles were so inserted before or after one to eight photoinductive cycles, floral expression was much more inhibited. Five short cycles inserted before the dark period of nine or ten photoinductive cycles did not materially inhibit initiation, but when inserted after the dark period, very few plants were flowering. In general, when ten short cycles were included the floral response ranged between these two.

EXPERIMENT 10.—This was designed to test the results obtained in experiment 8 as well as to compare the effectiveness of continuous light of low intensity with short cycles which included 3-minute light periods of low and high intensity in preventing floral initiation in soybean.

The plants were 30 days old at the start of the various treatments and had been under long photoperiod in the greenhouse for that entire time. The same precautions employed before and after treatments in other experiments were used here. Table 8 gives the relative humidity and temperature readings made every 3 hours, and maximum, minimum, and averages for the five rooms used. Fluctuations in both temperature and relative humidity were gradual.

White fluorescent light (1200 foot-candles) was used for the 10-hour photoperiods, and similar light of 20 foot-candles was used for the low intensity con-

TABLE 8

Room	TEMPERATURE (° F.)			PERCENTAGE RELATIVE HUMIDITY		
	MAX.	MIN.	AVE.	MAX.	MIN.	AVE.
Darkroom.....	86	76	82	80	63	77
Continuous light (20 f.c.).....	88	79	84	80	47	75
Continuous light (1200 f.c.)...	89	79	85	85	53	75
Short cycle (200 f.c.).....	84	78	81	84	64	75
Short cycle (20 f.c.).....	83	76	80	87	64	75

tinuous light treatment. The 3-minute light periods of the cyclic treatment were of 20 and 200 foot-candles Mazda filament light.

On July 8, 1940, 800 soybeans were selected for uniformity. Twenty of these were dissected immediately and every node was found to be vegetative. Eighty more were designated controls and divided into four lots of twenty plants, each receiving one of the following treatments:

1. Continuous long photoperiod. All plants vegetative.
2. Ten photoinductive cycles consisting of 10-hour photoperiods and 14-hour dark periods, then returned to long photoperiod. All plants flowering at conclusion of experiment. Average number of nodes with flowers, 5.3.
3. Ten days of continuous light of 20 foot-candles, then returned to long photoperiod. All plants vegetative at conclusion of experiment.
4. Ten days of continuous light of 1200 foot-candles, then returned to long photoperiod. All plants vegetative at conclusion of experiment.

Two groups of 200 plants each were given ten cycles consisting of 10 hours of light, followed by 14 hours of continuous darkness, followed by a given number of short cycles of alternating 3-hour dark periods and 3-minute light periods. One group received a high-intensity light (200 foot-candles) during the 3-minute period,

while the other group received a low-intensity light (20 foot-candles) during this period. One lot of twenty plants from each group received 1, 4, 6, 8, 10, 11, 12, 13, 14, and 15 short cycles. The remaining plants were divided into fifteen lots of twenty plants, each lot receiving ten cycles of 10 hours of bright light (1200 foot-candles) plus 14 hours of continuous darkness plus a given number of hours of continuous light at 20 foot-candles. One lot received 3 hours of the low-intensity light

TABLE 9

EFFECT ON FLORAL INITIATION IN SOYBEAN OF SHORT CYCLES OF 3 HOURS OF DARKNESS AND 3 MINUTES OF LIGHT OF VARIOUS INTENSITIES OR CONTINUOUS LIGHT OF LOW INTENSITY FOLLOWING EACH DARK PERIOD OF TEN PHOTOINDUCTIVE CYCLES. DATA TAKEN 21 DAYS AFTER COMPLETION OF TREATMENT. EACH LOT OF TWENTY PLANTS

No. OF SHORT CYCLES FOLLOWING EACH DARK PERIOD OF PHOTOINDUCTIVE CYCLE	INTENSITY OF LIGHT OF SHORT CYCLES								CONTINUOUS LIGHT (20 F.C.)*			
	20 F.C.				200 F.C.							
	No. OF PLANTS			AVE. NO. NODES WITH FL.	No. OF PLANTS			AVE. NO. NODES WITH FL.	No. OF PLANTS			AVE. NO. NODES WITH FL.
	FL.	VEG.	DEAD		FL.	VEG.	DEAD		FL.	VEG.	DEAD	
1. ....	20	0	0	4.2	19	1	0	4.1	20	0	0	3.5
2. ....									2	17	1	2.5
3. ....									0	20	0	0.0
4. ....	5	14	1	2.0	0	20	0	0.0	0	19	1	0.0
5. ....									1	19	0	2.0
6. ....	6	11	3	2.5	4	14	2	1.8	9	10	1	1.7
7. ....									5	13	2	2.0
8. ....	9	4	7	4.0	15	2	3	3.5	3	12	5	2.7
9. ....									0	14	6	0.0
10. ....	1	6	13	2.0	1	11	8	2.0	0	13	7	0.0
11. ....	0	5	15	0.0	1	9	10	2.0	0	11	9	0.0
12. ....	0	3	17	0.0	0	0	20	0.0	0	6	14	0.0
13. ....	0	2	18	0.0	1	3	16	3.0	0	4	16	0.0
14. ....	3	3	14	4.0	0	2	18	0.0	0	12	8	0.0
15. ....	1	2	17	3.0	0	0	20	0.0	0	6	14	0.0

\* Each short cycle represents 3 hours of continuous light.

following the dark period, a second received 6 hours, a third 9 hours, etc., to a maximum of 45 hours.

A number of the plants of some treatments did not survive the experimental period. Apparently this was due to the relatively short length and low intensity of the 10-hour photoperiods which made up each photoinductive cycle. The results of experiment 10 are shown in table 9.

The results of experiment 10 correspond in general to those obtained in experiment 8 (figs. 3, 5). Relatively short alternating periods of light and darkness, as well as continuous light of low intensity, when inserted after the 14-hour dark

period of the photoinductive cycle may reduce the photoinductive effect of such a cycle. The effectiveness of such treatment appears rhythmical (fig. 5). There is a rapid decrease in the number of plants flowering when 2-4 short cycles were so in-

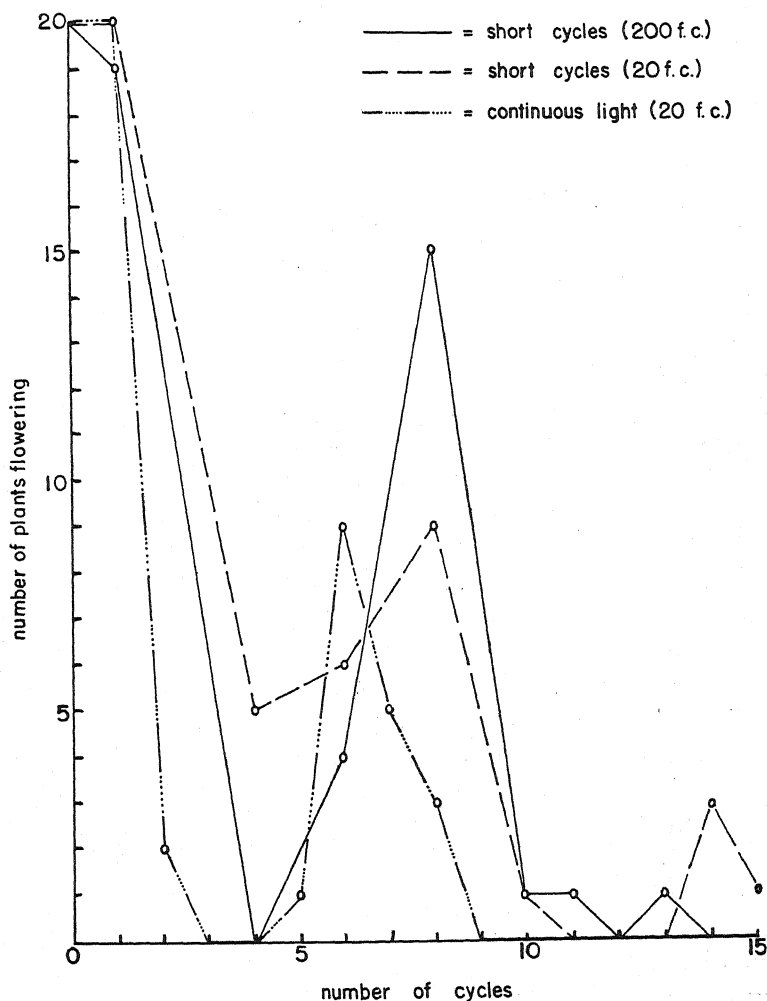


FIG. 5.—Effect on initiation in Biloxi soybean of ten photoinductive cycles which include short cycles (3 hours of darkness and 3 minutes of light or 3 hours of continuous light of low intensity) inserted after 14-hour dark period. Each short cycle represents lapse of 3 hours. Data taken 21 days after completion of treatment.

serted, then a rapid increase in the number flowering (5-9 short cycles), which is followed by another decrease. An insufficient number of the plants receiving 10-15 short cycles survived to warrant a definite statement as to their effect;



however, there is indication that an increase in the number flowering would have occurred. The average number of nodes per plant with flowers and the total number flowering of the various treatments roughly follows the same type curve (figs. 3-5). Whenever short cycles were introduced into the photoinductive cycle, regardless of their number or their place of insertion, fewer nodes per plant had flowers than occurred on plants subjected to a corresponding number of photoinductive cycles but without the short cycles.

### Discussion

These results substantiate previous findings (1, 3, 6, 7, 9) that in both cocklebur and Biloxi soybean photoperiodic induction takes place as a result of interaction between conditions brought about during exposure both to light and to darkness. This cyclic alternation of light and dark periods, when of appropriate duration, intensity, and frequency, results in photoperiodic induction. Under certain of these conditions cocklebur requires one such photoinductive cycle, and Biloxi soybean three, for photoperiodic induction. The necessary duration of the light and the dark period is dependent upon a number of factors, such as intensity and quality of light, temperature, age of plants, age and position of leaves, and so on (1-10). At present the nature of the interactions which result in photoperiodic induction are unknown.

The results of the present work indicate that cocklebur, when exposed to relatively bright light, may undergo certain changes or reactions which so alter the plant that if it is immediately exposed to a relatively long dark period it becomes photoperiodically induced and may subsequently initiate floral primordia. If a plant which has been exposed to such a period of bright light is exposed to relatively short alternating periods of light and darkness, however, the effectiveness of the photoperiod disappears, and even though subsequently subjected to a long dark period, initiation does not occur. The same results brought about by these relatively short alternating light and dark periods may also be produced by exposing a plant to continuous light of very low intensity, although in the latter instance the time required for the apparent lowering of the effectiveness of the previous photoperiod is much longer.

During photoperiods of bright light the establishment of A may occur at a rapid rate and there may result a higher degree of its effectiveness at the end of the photoperiod than at the beginning. Similarly, during photoperiods with low-intensity light the rate of establishment of A may be lower, and in complete darkness or during photoperiods with a very low light intensity its rate of establishment may actually be lower than the rate of its destruction, resulting in a lower level of effectiveness at the end of the period than at the beginning. If such were the case, it would be expected that a low temperature during treatment with

short cycles would result in a lower rate of decrease of the conditions brought about during the photoperiod. Such does not seem to be the case, since variations in temperature produced only slight differences in the number of short alternating cycles of light and dark periods necessary to prevent initiation.

Whatever the reasons, it seems apparent that conditions prevailing during the photoperiod influence the relative effectiveness of A. This is indicated by the preceding results, which show that a greater number of short cycles must be used to prevent photoperiodic induction following a period of bright light than following a period of relatively low light intensity; and that following a series of eighteen alternating cycles of short light and dark periods the length and intensity of photoperiods determine the subsequent return to a condition resulting in photoperiodic induction. It would appear also that the number of short cycles needed to prevent photoperiodic induction might serve as a measure of the accumulated effectiveness of A during previous exposure to light. Utilization of this method may give a quantitative basis for the measurement of the rate and degree of reaction on the part of the plant to exposure to photoperiods at various light intensities, temperatures, etc. Such a procedure has been carried out, and the results indicate that whatever the conditions brought about during exposure to the photoperiod, they take place to a greater degree during photoperiods of high light intensity than during those of lower intensity. A low temperature during the photoperiod apparently results in a slower rate of establishment of the effectiveness of A. These results are in general agreement with the response of Biloxi soybean to experiments of a similar nature and indicate the similarity in response between the two plants.

These results with cocklebur and Biloxi soybean may lead to a possible interpretation of apparently conflicting results obtained by other workers. It has been noted from time to time that photoperiodically sensitive plants may respond differently, depending upon the time of day that they receive supplementary illumination. Thus it has been the general practice, when it was desirable to expose plants to long photoperiodic conditions, to expose them to natural daylight and supplement this by continued exposure to light from Mazda lamps into the night. Contrary to this general practice, the supplementary illumination has occasionally been given from a designated time during the night until sunrise. In one case supplementary illumination followed the photoperiod of high light intensity and in the other case preceded it. A possible explanation of the conflicting results may be that with certain plants the photoperiodic induction more readily results if a period of bright illumination immediately precedes exposure to darkness. In this connection, the experiments with Biloxi soybean are of interest. While the short cycles were partially effective in inhibiting initiation, whether they immediately preceded or immediately followed the dark period, there was considerable difference in their relative effectiveness—not only with reference to the number of

plants flowering but also with reference to the number of nodes per plant which possessed floral primordia. More evidence is needed before definite conclusions can be reached, but preliminary results indicate that treatment immediately following the dark period would prove most effective.

The indications which were obtained as to the cyclic changes in sensitivity on the part of the soybean are difficult to interpret, but it would seem that utilization of cycles other than those based on 24 hours may bring about ambiguous results because of cyclic changes in sensitivity on the part of the plant which seem to follow a 24-hour cycle. It may be that future work should involve treatment of the plants from the time they are planted until the end of the experiment to avoid such conditions.

The results of experiments with both cocklebur and Biloxi soybean at least indicate that a practical method of maintaining short-day plants in the vegetative condition would be to expose them to brief periods of illumination during the night. This should prove even more effective in maintaining the plants in a vegetative condition than the common practice of exposing them to continuous illumination of low light intensity during a part of the night.

### Summary

1. In cocklebur, the conditions brought about during the photoperiod may be diminished to such a point that plants fail to initiate floral primordia when subsequently subjected to a 12-hour dark period. The methods employed are the insertion between the photoperiod and the dark period of the photoinductive cycle of a given number of short alternating periods of darkness and light or of a given number of hours of continuous light of low intensity. When Biloxi soybean was treated in the same manner there was no reduction in the number of plants flowering, although there was a decrease in the number of nodes with flowers.

2. In Biloxi soybean, short cycles of light and darkness, or continuous light of low intensity, when inserted following the dark period of each photoinductive cycle, reduced considerably the number of plants flowering as well as the average number of nodes per plant with flowers. This response appears rhythmical, and there are indications that utilization of cycles other than those under which the plant has been growing may bring about ambiguous results.

3. Effective short cycles consisted of either 3 hours of darkness and 3 minutes of light or 2 hours of darkness and 2 minutes of light.

4. The use of short alternating periods of darkness and light may be a more economical method of maintaining "short-day" plants in a vegetative condition than the common practice of exposing them to continuous supplementary illumination of low intensity.

5. The intensity of the light during these short periods apparently affects the response; and in cocklebur the temperature prevailing during the cyclic treatment had little, if any, effect.

6. A range of conditions imposed during the photoperiod of a photoinductive cycle can be directly correlated with the extent or degree of floral initiation shown by cocklebur subjected to the various treatments.

7. Following eighteen short cycles, cocklebur may regain the capacity of being induced and develop flowers if subjected to three photoinductive cycles consisting of photoperiods of 2 or more hours at 2000 foot-candles or 10 hours of 100 foot-candles and 14 hours of continuous darkness. Moderate to high temperatures during the photoperiod and an increase in the intensity and duration of the light favor establishment of the conditions brought about by the photoperiod as measured in these experiments.

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# EFFECT OF GROWTH SUBSTANCES ON THE ABSCISS LAYER IN LEAVES OF COLEUS<sup>1</sup>

R. MAURICE MYERS

(WITH NINE FIGURES)

## Introduction

LAIBACH (11) reported that orchid pollinia inserted in the split end of petioles of debladed leaves of coleus and certain other plants delayed abscission of the petioles 11-20 days longer than that of untreated petioles. LA RUE (12) found that indoleacetic acid (heteroauxin), pollen, and urine delayed petiole fall for a few days when they were applied to debladed petioles of coleus leaves. A study of the effect of growth substances in relation to other factors known to affect abscission seemed desirable.

The earlier literature on abscission is extensive and will not be discussed here. It is reviewed by KENDALL (9), LLOYD (13, 14, 15), and SAMPSON (18). It is generally agreed that abscission is usually preceded by the development of an absciss layer followed by the disintegration of certain lamellae in the walls of cells in this layer. SAMPSON found that an absciss layer started to form in the third pair of leaves below the terminal bud of coleus plants bearing eight pairs of leaves, and that the layers were not mature until these leaves were the sixth pair below the terminal bud. He found that at the time of abscission the lamellae of the primary wall adjacent to the middle lamellae disintegrated before the middle lamellae. The cellulose properties of these lamellae disappeared and a test for pectic compounds became more intense. These facts led him to describe abscission as a change of cellulose to pectose and later to pectic acid which resulted in an excess of pectic acid over the amount of calcium necessary to maintain the solidity of the middle lamellae of the cell wall in the absciss layer. His inferences as to the nature of the changes in cellulose, however, were made in the absence of adequate chemical data. Even today the changes that may occur in these compounds in cells under the action of enzymes are not well enough known to admit a more detailed description.

## Material and methods

Several thousand plants of *Coleus blumei* Benth (var. Golden Bedder) were propagated from cuttings in a greenhouse during the winter and spring. Since coleus has opposite leaves, certain treatments can be performed on one leaf while

<sup>1</sup> A fuller account of this research is available, see 17.

the other of the same age is left as a control. At any particular time the plants usually have eight pairs of leaves, not counting those folded together in the terminal bud. Eight to ten plants were used for each experiment, and each experiment was repeated several times.

A number of different treatments were used and these are described separately. The growth substances used were carefully weighed and mixed with the quantity of anhydrous lanolin necessary to make the desired concentrations. The mixtures were heated until the lanolin melted and the substances were completely dispersed. Fresh mixtures were made at frequent intervals, although it was found that the growth substances remained active in the lanolin for several years. The amount of paste applied was not measured, but about the same amount was applied to each part of the plant. Controls were treated similarly with anhydrous lanolin, and all cut surfaces were coated with lanolin to prevent desiccation.

Data for the pre-abscission period were collected daily. The pots containing the plants were tapped gently at that time, as petioles in which abscission was practically complete often remained attached to the stem for several days by a small piece of dried tissue.

The internal changes were followed by the use of freehand and rotary microtome sections. Freehand sections were frequently employed because the changes taking place during abscission could be better observed. Longitudinal sections cut through the stem and petioles at each node gave comparable views of the absciss layers of the pairs of opposed leaves.

### Investigation

#### GROWTH OF LEAVES

In order to discover any possible relationship between development of the leaves, growth of the absciss layer, and subsequent abscission, the relative rates of development of the different parts of a leaf were measured.

All leaves on several plants were marked off into 4-mm. squares with a special rubber stamp. As a check, leaves of other plants were marked off in 4-mm. segments with a small brush and India ink. Microscopic examination of the cells of the petioles, especially those close to the absciss layer, were also made.

The growth of a coleus leaf (fig. 1) is similar to that of tobacco as described by AVERY (1), but it is more basipetal. There is an increasing gradient of expansion from the tip of the leaf to the base of the blade. A similar gradient of expansion occurs in the midrib and petiole, almost to its base. By the time a leaf has attained one-fifth its final size the tip has practically ceased expanding. The greatest expansion is in the second and third pairs of leaves below the terminal bud; considerably less in the fourth pair; and in the fifth, expansion of the blade has ceased but the petioles of these leaves and the sixth and seventh pairs of leaves continue to

elongate. The base of the petioles distal to the absciss layer has the longest period of expansion of the entire leaf, although it does not elongate as much as the segments of the petiole immediately above it. The base of the blade has the greatest amount of expansion, which is attained before elongation of the petiole has ceased.

Growth of the absciss layer begins in the partially enlarged and vacuolated cells in the base of the petiole of the third pair of leaves below the terminal bud and continues until these leaves have become the sixth pair. The blade is 85-90 per cent expanded before the absciss layer begins to develop. There is considerable cell enlargement in the petiole distal to the absciss layer. The cells become larger than those of the stem cortex at the base of the petiole. The epidermal cells distal to the absciss layer become greatly elongated and do not increase much in width. The base of the petiole proximal to the absciss layer and a part of the stem cortex contributes little to the increase in length of the petiole.

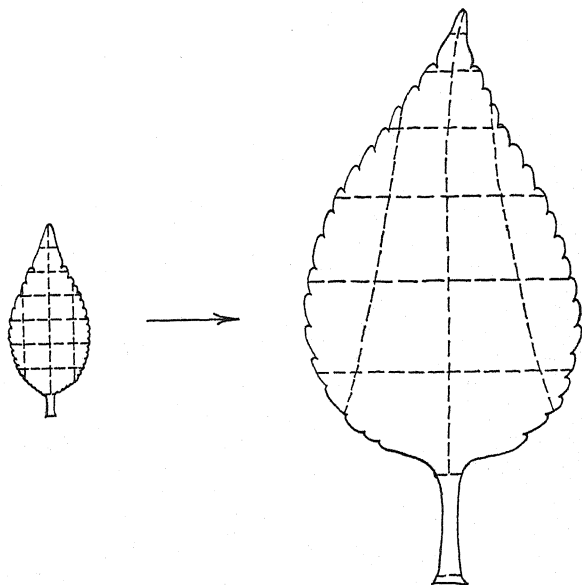


FIG. 1.—Place of expansion in growth of typical coleus leaf

#### DEVELOPMENT OF ABCISS LAYER AND ABCISSION

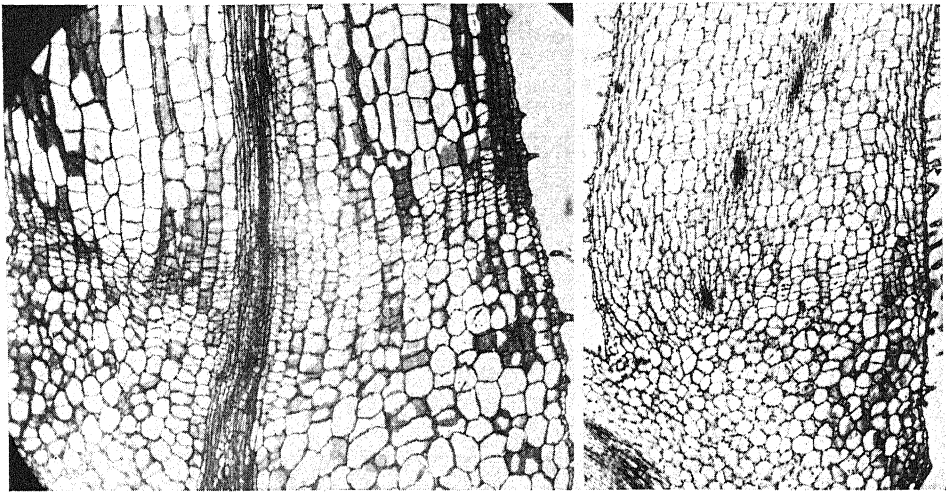
Under ordinary conditions the loss of leaves of coleus probably is always the result of two processes: growth of an absciss layer, followed by dissolution of certain lamellae in the cell walls in this layer. Under certain conditions, either process may occur without the other.

The absciss layer is formed at the base of the petiole in a plane normal to the opposed leaves. It usually begins development in the third pair of leaves and matures in the sixth and seventh pairs. Its initiation is first evident by the appearance of a few transverse divided cells in the epidermis and periphery of the cortex. In a short time it appears as a ring of dividing cells around the base of the petiole. These cells continue to divide, and as more cells become involved the ring gradually widens and thickens, finally extending across all the tissues of the petiole except the xylem. Ten to fifteen layers of cells may be involved in the



outer part, but only six to eight layers in the central part of the petiole. The absciss layer, however, is about the same thickness throughout, since the cells are of different sizes.

When sections are cut at right angles to the plane of the absciss layer it has the appearance of a meristem. The cell contents stain darker than the other cells in the cortex, have more prominent nuclei, and lack large vacuoles. As it nears maturity the cell contents become granular and the cell walls less distinct. At maturity it becomes externally evident by the slight depression in the petiole (the



FIGS. 2, 3.—Fig. 2 (left), longisection through base of petiole of sixth pair of leaves below terminal bud, showing mature absciss layers. Fig. 3 (right), same of petiole of first pair of leaves below terminal bud; leaf debladed 3 months previously.

abscission zone), since the cells on either side of it continue enlarging while those in the absciss layer divide with but little enlargement.

In the younger leaves the cells in the region where the absciss layer will later form are indistinguishable from the other cortical cells of the petiole. Beginning with the third pair of leaves below the terminal bud, however, a distinct difference becomes evident. The cells of the petiole distal to the absciss layer become much larger and more elongated than those of the abscission zone and stem cortex. An absciss layer that is mature is shown in figure 2.

The final step in the separation of the leaf from the stem is the dissolution of a portion of the walls of some of the cells in the absciss layer. Shortly before abscission takes place, the petiole—and eventually the entire leaf—begins to lose its green color, and at the time of abscission it is usually pale yellow. When a break in

the lower part of the base of the petiole is evident the abscission processes are well advanced, and in a few days separation is completed.

Usually disintegration of cell walls is evident in two or three layers in the distal part of the absciss layer. The process apparently begins in the cells in the lower side of the petiole. As the middle lamellae of these cells dissolve, the longitudinal shrinkage of the cortex pulls them slightly apart and their exposed surfaces round up as a result of turgor. Once the first break is made the dissolution continues rather rapidly. Dissolution continues through the absciss layer and gradually extends through the epidermal cells and through the cortex, until all that supports the petiole is the upper part of the cortex and xylem. Finally these cortical cells separate, the xylem ruptures, and the leaf falls.

#### EFFECT OF REMOVING THE BLADES

Investigators (3, 4, 7, 19, 15, 18, 20, 21, 22, 23) have shown that abscission is accelerated by high and low light intensities, different lengths of day, high and low water supply, high and freezing temperatures, low concentrations of anesthetics, toxic concentrations of acids and salts, ethylene gas, wounding of and complete removal of the blade.

To follow the internal changes that occur after deblading and to compare these changes with those occurring in an intact leaf, all the blades of one group of plants were removed and in a second group one blade of each pair of leaves was removed, the opposite leaf remaining intact. Sections cut through the absciss layers of the younger leaves usually indicated that cell division of debladed petioles had been accelerated. In the fifth and lower pairs of leaves acceleration of cell division was not so evident. In these older leaves the absciss layers were mature or almost so, and deblading did not cause additional cell division. The deblading, however, always accelerated abscission of the petioles.

There were several variations. Occasionally abscission occurred without development of the absciss layer. Abscission of debladed petioles of the third, fourth, and fifth pairs of leaves often occurred without further development of the immature absciss layers, beyond that of the opposed intact leaves. The cell walls in the debladed petioles were less distinct, the cell contents more granular and more readily stained—the usual conditions just prior to abscission. In the first petiole below the terminal bud, in which no absciss layer was visible at the time of deblading, abscission often took place without development of an absciss layer. It was frequently observed that these young petioles did not increase in size and did not abscise after deblading, or abscised only after more than 3 months, when they were in the same position as the sixth pair of leaves. In such petioles a well-defined absciss layer had developed (fig. 3), but dissolution of the cell wall lamellae was not taking place. The factors causing this long delay have not been discovered.

After deblading, the youngest petioles did not abscise as soon as the oldest petioles, and there was a gradient in time between them (fig. 5). At the time of deblading the absciss layers were in various stages of development, from none in the first two pairs of leaves to one that was mature in the sixth pair. Apparently the length of time before abscission under these conditions is related to the degree of maturity of the absciss layer or to the age of the leaf. Since deblading usually accelerates development of the absciss layer, it matures more rapidly in older leaves where it is already well advanced. DOUBT (3) found that treating coleus with ethylene gas caused the oldest leaves to abscise first.

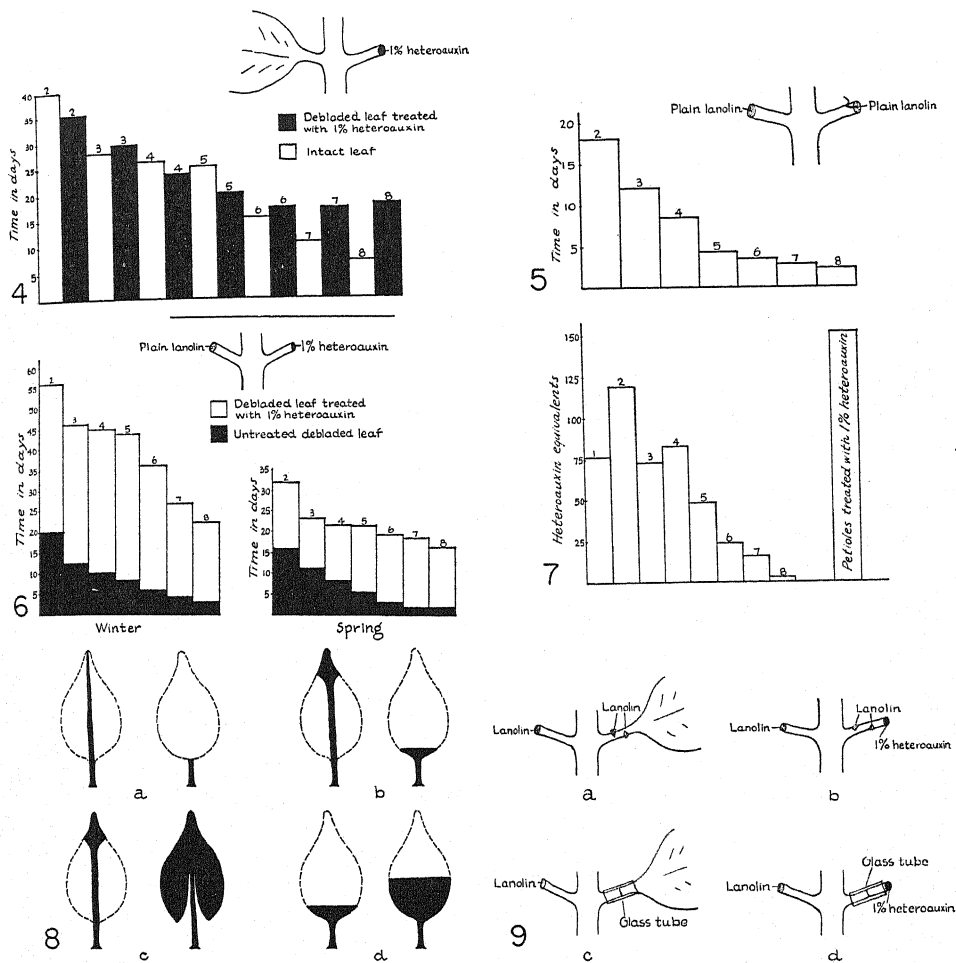
EFFECT OF GROWTH SUBSTANCES ON DEVELOPMENT  
OF ABSCISS LAYER AND ON ABSCISSION  
OF DEBLADED PETIOLES

In one group of plants a blade was removed from one of two opposite leaves at each node of the stem and a 1 per cent mixture of heteroauxin applied to the ends of the debladed petioles (fig. 4). By cutting freehand sections it was possible to compare the absciss layers and abscission phenomena in a treated debladed petiole with those in the petiole of the opposite intact leaf.

Development of the absciss layers in the treated debladed petioles was similar to that in the petioles of intact leaves. The time elapsing before abscission occurred was nearly the same (fig. 4), except in the oldest leaves which dropped 1-10 days sooner than the treated debladed petioles. As will be shown later, this fact may be correlated with the smaller amount of growth substance coming from the blades of the older leaves, indicating that heteroauxin delays the final stages of abscission.

In another experiment all the blades were removed from the plants and a 1 per cent heteroauxin mixture was applied to one petiole at each node, and the opposite petiole smeared with plain lanolin. Treatment of the debladed petioles usually delayed development of the immature absciss layers, and abscission. It retarded initiation of the absciss layer and its growth at any stage of development. The increase in the pre-abscission period depended upon the stage of development of the absciss layers and the time of year (fig. 6). The more mature the absciss layer the shorter the time before abscission. During May the abscission time was less than that for the leaves debladed in January. In the sixth, seventh, and eighth pairs of leaves the absciss layers were so well developed that there was little difference in their appearance in the treated and untreated petioles. Usually there was decided delay in the growth of the absciss layers in the young petioles, but occasionally no difference could be seen. These facts indicate that either the heteroauxin did not always delay development of the absciss layer, or the development of the absciss layer was not always accelerated by removal of the blade.

Deblading a leaf stopped elongation of the petiole. Diameter measurements were not made, but it was apparent that there was little or no increase in diameter.



FIGS. 4-9.—Fig. 4, pre-abscission period of pairs of untreated debladed petioles; numbers refer to position of leaves below terminal bud. Started March 2. Fig. 5, same of treated debladed petioles and opposed intact leaves. Started May 2. Fig. 6, same of treated debladed petioles and opposed untreated debladed leaves. Started in January and in May. Fig. 7, amount of growth substance diffusing from leaves and petioles treated with 1.0 per cent heteroauxin; tests carried out in March. Average of 10 tests. (In gammas of heteroauxin equivalents; controls with agar containing 100 gammas of heteroauxin per liter.) Fig. 8, method of treating leaves (dotted lines indicate portion removed). Fig. 9, method of treating leaves and petioles.

A treated debladed petiole, however, usually elongated as much as the petioles of the opposite intact leaf. Likewise the increase in diameter was similar. MAI (16)

had similar results when he treated debladed petioles with pollen. The amount of enlargement depended on the age of the leaf. Many of the younger petioles continued to elongate and reached or exceeded the dimensions of the petioles of the opposite intact leaves. The petioles of the first leaves below the terminal bud did not enlarge as much as the petioles of the second and third leaves. There was little elongation of the older petioles, but there was usually more than that in the opposite intact leaves.

These experiments indicate that the heteroauxin has an effect on elongation of the petioles, development of the absciss layers, and on abscission of the petioles similar in kind to that of the blade and nearly equal to it in magnitude.

In other experiments concentrations of 1, 0.5, and 0.1 per cent heteroauxin were used. The 1 and 0.5 per cent concentrations were about equally effective in delaying abscission but about 25 per cent more effective than the 0.1 per cent concentration.

Other growth substances, indolepropionic and indolebutyric acids, were applied to debladed petioles in 1 per cent concentrations. The indolebutyric acid was about as effective as heteroauxin in delaying abscission, but the indolepropionic acid was about 25 per cent less effective.

#### MEASUREMENTS OF GROWTH SUBSTANCES DIFFUSING FROM LEAVES

Measurements of growth substances diffusing from coleus leaves were made by the double decapitation *Avena* technique (24). The leaves were severed from the plants, the cut ends wiped with moist filter paper and attached to a sheet of agar with a drop of 0.5 per cent gelatin. At the end of 2 hours the leaves were removed, the agar allowed to stand for 40 minutes and then cut into twelve equal blocks and applied to decapitated *Avena* coleoptiles. Tests were made for the amount of phytohormones diffusing from petioles treated with 1 per cent heteroauxin on the distal ends.

The greatest amount of phytohormones diffused from the upper leaves (fig. 7). It is in these leaves that expansion occurs most rapidly. There was less diffusing from the older leaves where expansion had almost ceased. Usually there was none diffusing from the eighth pair of leaves. AVERY (2), working with tobacco, and GOODWIN (5) with two species of *Solidago*, had similar results. The quantity of substances diffusing from the treated petioles exceeded that coming from untreated leaves but was not much greater than that coming from the second pair of leaves during the spring.

Leaves tested during the winter and early spring gave very weak or negative tests for growth substances; during the late spring the amount increased considerably. GUSTAFSON (6) had similar results with certain fruits. It is interesting to note that abscission took place more slowly during the winter when the amount of

phytohormones was low than during the late spring when it was higher, and that a 1 per cent concentration was more effective in inhibiting abscission in the summer than in the winter. Treated petioles remained on the plants much longer in the winter than in the summer, but the effectiveness of the treatment is based upon the behavior of the controls for each season.

During the winter (January to March) the second untreated debladed petioles abscised in an average of 21 days, and the treated debladed petioles abscised in an average of 57 days (fig. 6). In the spring (May to June) similar petioles abscised in 16 and 32 days, respectively (fig. 6) and in the summer (June to August) in 4 days and 30 days, respectively. The abscission of intact leaves was considerably slower during the winter than in the spring and summer. During the winter months growth of the entire plant was also much slower than during the spring and summer. It is obvious that internal conditions other than the growth substances are also primary factors in abscission.

#### MOVEMENT OF THE INHIBITING EFFECT

Experiments were designed to determine whether the inhibiting effect of the blade moved across, up, or down the stem. In one group of plants one blade of each pair of leaves was removed, the opposite leaf remaining intact, and in another group of plants both blades were removed. There was no significant difference in the time of abscission or in the development of the absciss layers in the debladed petioles of the first group and in the second group. This indicates that there is no movement of the inhibiting effect of the blade across the stem to the absciss layer of the opposite leaf, at least in significant amounts.

In another experiment all blades were removed from two groups of plants. All petioles of one group remained untreated except for a coating of plain lanolin. In another group one petiole of each pair was treated with 1 per cent heteroauxin, the opposite petiole being smeared with plain lanolin. There was little difference in the development of the absciss layers or in the time of abscission of the untreated petioles of these two groups.

One per cent heteroauxin placed on the sides of the stem of plants from which all the blades had been removed did not cause significant inhibition of the debladed petioles, either above or below the treated part. It has been shown by SNOW (19) and others that under natural conditions the movement of growth substances is polar, that is, down the stem. AVERY (2) showed that phytohormones moved down the leaf through the veins and midrib. HITCHCOCK and ZIMMERMAN (8), however, showed that the application of certain synthetic substances in water to the soil of potted plants caused root formation on the stem, epinasty of leaves, and other effects, indicating a movement up into the stem and leaves. They were unable to secure these effects, except near the point of applica-

tion, when they used the mixture in lanolin. When cuttings were placed in a strong solution of heteroauxin (1:30,000) for 4 hours and the blades removed there was delay of a few days in the abscission of the petioles as compared with comparable cuttings in distilled water.

When approach grafts were made by splicing one of a pair of debladed petioles to an intact leaf of another plant, abscission of the grafted petioles was delayed not more than 2 days longer than the controls, although both petioles usually abscised in about the same time. This may indicate some movement of plant hormone across the graft. No grafts formed a union.

These data indicate that there is usually no significant movement of the inhibiting effect of the blade or of applied substance across or up or down the stem. When cuttings are placed in a strong heteroauxin solution there is apparently some movement up the stem and into the petioles.

Later evidence will show that the inhibiting effect of applied hormone, and presumably also of the blade, is correlated with the amount of substance translocated. Further evidence will be given indicating that under certain conditions there is no significant movement of the inhibiting effect from an intact leaf to the opposite intact leaf.

#### EFFECT OF PLACING PLANTS IN CONTINUOUS DARKNESS

ZIMMERMAN and HITCHCOCK (25) found that tomato plants kept in the dark for 5 days did not show geotropic effects, but curved down when heteroauxin was applied to the stem. They mentioned that if the assumption of KEEBLE *et al.* (10)—that the upward growth of plants placed in a horizontal position is due to a redistribution of substance—is correct, plants that no longer grow upward should not contain growth substances.

AVERY (2) reported that phytohormones disappeared from tobacco plants after 5 days in the dark. ZIMMERMAN and HITCHCOCK (25), however, reported that tobacco plants 18 inches tall still elongated after 30 days in the dark. They found it difficult to account for this continued growth if there can be "no growth without the presence of hormones." Using plants with storage organs (potatoes), they obtained geotropic effects and stem elongation after more than 60 days in the dark. Starch and sugars were detected as long as growth continued.

Rapidly growing coleus plants were placed in a dark box in June. At the end of about 10 days the leaves began to abscise, the youngest and oldest leaves abscising first. No absciss layers were found in the upper two pairs of leaves at the time of abscission. In the older leaves there was apparently some acceleration of these layers. Abscission of intact leaves was delayed by the application of heteroauxin 1-10 days longer than the opposite intact untreated leaves. There was usually more elongation of the petioles and more increase in area of the blade of the

younger intact treated leaves than in the opposite intact leaves. The untreated plants had lost most of their leaves after 21 days, but the stem and remaining petioles were still elongating and grew upward when placed in a horizontal position. Application of growth substances also caused bending of the stem. Shortly after this the plants began to rot and the experiment was discarded. Decay usually took place at the tip of the stem and in leaves that had not abscised. It affected entire leaves but ceased at a point where the absciss layer usually formed. It did not involve any stem tissue until later.

Coleus plants were placed in shaded parts of the greenhouse. One of each pair of leaves at the five lower nodes below the terminal bud was inclosed in a black sateen bag,<sup>2</sup> the opposite leaves being inclosed in similar white bags. The leaves in the black bags abscised 1-4 weeks before the leaves in the white bags. Other plants with the leaves similarly inclosed in white and black bags, and not in either type of bag, were placed in a dark box. The leaves of these three groups of plants abscised in about the same time. These data indicate that the blade is most effective in delaying abscission when it is exposed to light. The effect is not translocated from a leaf in the light to the opposite one inclosed in a black bag in sufficient quantities to cause a significant delay in abscission.

#### EFFECT OF REMOVING CERTAIN PORTIONS OF THE BLADE

LA RUE (12) reported that as little as one-fourth of the blade of a coleus leaf left intact was sufficient to inhibit abscission of the petiole, but he did not indicate the part of the blade removed or the extent of the inhibition. In a series of experiments to check the relative effectiveness of different parts of the blade, various parts and amounts were removed. Plants bearing seven pairs of leaves were used in each experiment:

1. At each of the five lower nodes one leaf of each pair was debladed (fig. 8a). At each of the five lower nodes, therefore, there was a debladed petiole opposed by a petiole plus the midrib. A narrow ridge of the blade left attached to the midrib of the younger leaves expanded considerably, so the two upper pairs of leaves were not used. The pre-abscission period of petioles bearing the midribs was usually 1-2 days longer than the debladed petioles.

2. All the blade except a small portion of the tip was stripped from the midrib of one leaf at each of the five lower nodes. All the blade of the opposed leaf was removed except a portion at the base of the blade about equal in area to the tip of the opposite leaf (fig. 8b). At each node, therefore, a leaf with only the extreme tip of the blade intact opposed a leaf with a small portion of the base of the blade intact. The base of the blade delayed abscission  $1\frac{1}{2}$ -6 days longer than the tip

<sup>2</sup> A Weston light meter placed inside the bags gave no reading when held at a distance of 10 inches from a 60-watt bulb.



of the blade. The areas of the opposed leaves were equal only at the beginning of the experiment. The tips did not expand, but there was some expansion of the bases, especially of the younger leaves.

3. Incisions were made through the blade on each side of the midrib of a leaf at each of the five lower nodes. These incisions extended from the base of the blade almost to the tip, leaving an area at the end of the blade undisturbed and with the lower part of the blade detached from the midrib but connected to the tip of the blade (fig. 8c). The blades of the opposed leaves were removed from the midrib except for an area at the tips about equal to that of the undisturbed portion of the opposite leaf. The tip of the blade plus the rest of the blade detached from the midrib delayed abscission longer than the tip of the blade plus the midrib. The difference was less than 1 day for the older leaves to 5 days for some of the upper leaves.

4. One-half of the upper part of the blade of a leaf at each of the five lower nodes was removed. About seven-eighths of the upper part of the blade of the opposite leaves was removed, so that at these nodes a leaf with one-half the blade intact opposed a leaf with one-eighth the blade intact (fig. 8d). The larger portion of the blade delayed abscission longer than the smaller portions, the difference ranging from 6 to 8 days.

These data indicate that the base of the blade is more effective than the tip in delaying abscission. The midrib is not very effective. One-half the blade will delay abscission longer than one-eighth of the base of the blade.

#### EFFECT OF CERTAIN TREATMENTS OF THE PETIOLES

The following experiments were set up to compare the movement of the effect of the blade in delaying abscission with the movement of the effect of heteroauxin in delaying abscission:

1. Two deep incisions were made in the upper and lower sides of the petiole of a leaf at each node of the five lower nodes of coleus plants (fig. 9a). These incisions cut through most of the petiole's tissue and left only a narrow bridge of tissue connecting the two parts of the petioles and completely severed the semicircular vascular system. The blades were therefore attached to the petioles by a bridge of cells but without direct vascular connections. Lanolin was rubbed into the incisions and the leaves were supported in the usual position with a piece of tinfoil wrapped around the petioles. Usually the movement of water from the stem and leaves was greatly reduced by these incisions and the leaves soon wilted. The blade of the opposite leaf was removed. The debladed petioles abscised 2-10 days sooner than the leaves with the incised petioles. Some of the younger leaves par-

tially recovered and occasionally six to eight adventitious roots grew from the petiole, just distal to the upper incisions.

2. In a similar experiment all the blades were removed from a plant. Two deep incisions as described in (1) were made in a petiole at each of the five lower nodes. The distal ends of these petioles were treated with heteroauxin (fig. 9*b*); the opposite petioles left untreated. The debladed petioles abscised 3-14 days sooner than the opposed treated petioles.

3. The blades were severed from a leaf at each of the five lower nodes. These blades were then replaced on the stump of the petiole and held in place with a piece of glass tubing filled with lanolin or melted agar. The tube fitted tightly over the two portions of the petiole and held them close together (fig. 9*c*). The opposed petioles were debladed. There was no significant difference in the pre-abscission period of the debladed petioles and the petioles with the blade severed and replaced.

4. An experiment similar to (3) was set up with heteroauxin substituted for the blade. All the blades were removed from each of the five lower nodes. One petiole at each node was severed in the middle and replaced on the stump and held in place with a piece of glass tubing filled with lanolin or agar. The distal end of the petiole was coated with heteroauxin (fig. 9*d*). There was no significant difference in the time of abscission of the opposed petioles.

These data indicate that the movement of the effective agent of the blade in delaying abscission is similar to the movement of heteroauxin substituted for the blade, because: (a) the effect of the blades moves through the cells of the petiole when the vascular system is severed and the leaves wilted; (b) the heteroauxin delays abscission when it is applied to the end of a debladed petiole that has the vascular system severed; (c) the effect of the blade does not move across a cut surface in significant amounts, as a blade severed and replaced on the petioles does not delay abscission; and (d) the heteroauxin does not move across a cut surface in a debladed petiole that has been severed and replaced on the petiole, in sufficient quantities to delay abscission.

### Summary

1. Following the reports of others that growth substances and materials containing them delay abscission of debladed petioles, a study of their effects in relation to several other factors that affect abscission was made.

2. Growth of the coleus leaf is basipetal.

3. The blade is 85-90 per cent expanded before the absciss layer is initiated in the third pair of leaves and is mature in the sixth pair of leaves of plants bearing eight pairs.

4. Abscission usually occurs in the absciss layer, but under certain treatments it may occur without development of this layer.

5. Older leaves usually abscise before younger leaves, and factors that accelerate abscission cause older leaves to abscise first.

6. Removal of the blade accelerates abscission of all petioles, except the first pair of leaves. A well-defined absciss layer develops in these leaves after several months.

7. Partially debladed petioles do not abscise as soon as petioles from which the entire blade has been removed. Actively expanding portions of the blade are more effective in checking abscission than the mature parts.

8. The basal half of the blade delays abscission 7-10 days longer than the lower one-eighth of the blade, but not so long as the intact blade.

9. A greater amount of phytohormones diffuses from petioles of the upper leaves than from the lower leaves.

10. Tests for growth substances diffusing from leaves were negative or very low during the winter.

11. The upper intact leaves and the opposite debladed petioles treated with 1 per cent heteroauxin usually abscise in about the same time. The lower intact leaves abscise 1-7 days before the treated debladed petioles, indicating that heteroauxin delays the final stages of abscission in addition to delaying development of the absciss layer.

12. Abscission is more rapid in the spring and summer than in the winter.

13. Heteroauxin applied to debladed petioles has an effect similar to the blade in delaying abscission and favoring continued development of the petioles.

14. Development of the absciss layer and abscission is accelerated in leaves of plants placed in the dark. Heteroauxin inhibits the abscission of these leaves.

15. Heteroauxin will pass through debladed petioles which have been severed except for four or five layers of outer cortical cells in sufficient quantities to delay abscission for several days.

16. The effect of the blades passes through cells of petioles severed in a similar manner and delays abscission.

17. The pre-abscission period of debladed petioles of cuttings is increased by placing them in a strong solution of heteroauxin.

18. One and 0.5 per cent concentrations of heteroauxin are about equally effective in delaying abscission but are about 25 per cent more effective than a 0.1 per cent concentration.

19. Indolepropionic and indolebutyric acids have a similar effect in delaying abscission.

20. All experiments indicate that growth substances from the blades influence the development of the absciss layer and the process of abscission, but their relation to abscission phenomena does not seem to be a simple one.

The writer wishes to thank Dr. H. C. SAMPSON for suggesting the problem, for his critical advice, and for supervising the organization of the results.

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# EFFECT OF SOME ENVIRONMENTAL FACTORS ON FLORAL INITIATION IN XANTHIUM<sup>1</sup>

CONTRIBUTIONS FROM THE HULL BOTANICAL LABORATORY 517

LOUIS K. MANN

(WITH FIVE FIGURES)

## Introduction

Attention has recently been called to the fact that there are effects attributable to both the dark period and the photoperiod in the initiation of flower primordia and the development of flowers resulting from photoperiodic treatment. This interpretation has been applied to the reactions of short-day plants. In the cocklebur (*Xanthium pennsylvanicum*) and the Biloxi soybean (*Soja max*), the conditions prevailing during either the dark period or the photoperiod may be a limiting factor in the induction of flowers (2). For example, *Xanthium* will flower after exposure to a 12-hour dark period following a photoperiod of high-intensity light. Similar plants will remain vegetative if the photoperiod preceding this dark period is of very low light intensity. But, regardless of the length of the photoperiod or the intensity of the light, a continuous dark period of at least  $8\frac{1}{2}$ –9 hours is necessary for the induction of flower primordia.

In an endeavor to provide a brief formulation of the fact that both the photoperiod and the dark period are concerned in the ultimate conditions necessary for floral initiation and development, HAMNER (2) has suggested that their relationship may be represented as follows: A, B→C, in which A represents the changes or conditions which arise owing to exposure to light; B, those owing to darkness; and C, the possible summation or resultant changes related to A and B. These same terms are used here, but it is not assumed that definite, known reactions are represented by them. The physical or chemical changes or conditions constituting A, B, and C are still to be determined. Although there is some evidence that diffusible substances are involved (3), up to now attempts to isolate flower-inducing substances have not demonstrated either their actual occurrence or their nature.

Following specific photoperiodic treatments, conditions A, B, and their possible resultant, C, may become evident through the initiation and development of flower structures. In so far as A, B, and C pertain to floral initiation and develop-

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ment, the degree of initiation and development may be used as an indicator of the intensity of these conditions, although it is generally recognized that there are definite responses by other parts of the plant as well. In the following experiments conditions of light intensity and temperature were carefully controlled, and an estimate of the intensity of A and B as conditioned by these factors is assumed to be indicated by the degree of floral initiation and development.

### Material and methods

Experiments 1-4 and 8 were carried out in March, April, and May, 1940, at the U.S. Horticultural Station, Beltsville, Maryland. Seeds of *Xanthium pennsylvanicum* were collected at Chicago in the autumn of 1939 and stored out-of-doors during the winter. The seeds were planted in flats, the seedlings later transplanted to  $3\frac{1}{2}$ -inch pots, and most experimental treatments begun when the plants—carefully selected for height and vigor—were 40 days old. They were kept on greenhouse benches receiving natural daylight, which was extended from sundown until 2:00 or 3:00 A.M. by about 100 foot-candles of Mazda filament light.

The specific treatments usually required 4-5 days for completion, after which time the plants were returned to the greenhouse for the duration of the experiment, at the end of which the stage and type of development of the terminal bud of all plants were determined by means of a low-power binocular microscope. All dissections, unless otherwise noted, were made 22 days after the conclusion of treatment. Dissections of control plants were made before and after experiments to insure that conditions of long photoperiod had been maintained; that is, that plants which received no special treatment remained strictly vegetative. The plants grew vigorously, most of them being over 3 feet high at the time the terminal bud was dissected. Because of the limited amount of soil in each pot, the plants were watered with complete nutrient solution on alternate days from the time the treatment was completed until dissection.

The character of experiments 1-4 made necessary an extensive scale for recording floral stages, the frequently used divisions into inflorescence primordia, flower primordia, etc., not being sufficiently precise to indicate the several stages recorded. The degree of initiation or development of flowers in a plant was designated by a number. The stage of floral development of strictly vegetative plants was designated as zero. In plants not strictly vegetative the number was, in general, equivalent to the transverse diameter of the terminal staminate inflorescence measured in units of 0.1 mm. For diameters below 0.7 mm. the diameter alone was not an adequate measure, and six stages—based mainly on appearance—were recognized. An inflorescence with a diameter of less than 0.7 mm. usually possesses few, if any, flower primordia, but consists for the most part of a smooth,

bulbous inflorescence axis surrounded at the base by several primordia of bracteal leaves. When an inflorescence has reached the diameter of 0.7 mm. there are usually one or more cycles of flower primordia around the basal portion of the axis. As the inflorescence continues to develop, primordia appear in acropetal succession until the axis is completely covered, a condition found in almost all inflorescences of a diameter of 1.2 mm. or larger. For any given inflorescence diameter within the range 0.7–1.2 mm., the average relative portion of the axis covered with flower primordia was determined. If in any plant with an inflorescence diameter within this range the relative portion of the enlarged axis tip covered with primordia was greatly different from the average for that diameter, the measured diameter was modified slightly. For inflorescences with a diameter greater than 1.2 mm. the diameter was used directly. Since the environment during any experiment affects initiation and rate of floral development, the stage is of comparative value and its use is justifiable only within a single experiment, or in experiments conducted under identical conditions.

For any specific treatment within an experiment, a unit which usually consisted of ten or more plants was used. When all plants of a unit were flowering (no vegetative plants present), the average of their floral stages presents a fair estimate of the resultant effects of the stimulus. If, however, a unit was given a photoperiodic treatment of just sufficient strength to bring about the first recognizable stage of flowering, the variability in the response of these plants is such that approximately half of them would show various stages of flowering while the other half would remain vegetative. Among the vegetative plants, some would require only slight additional stimulus to bring about initiation of flowers, while others would require a much greater stimulus. Since these vegetative plants are all given the value zero as their stage of flowering, no negative values being used, the numerical average of the plants in the unit is much too high. As another example, suppose that following a given photoperiodic treatment one plant flowers out of a group of ten, the remainder being vegetative; the average stage of flowering of this group would be positive and equal to one-tenth the flower stage of the single flowering plant. Rather than make an arbitrary correction for these vegetative plants in the following graphs, a list of all units containing two or more vegetative plants is given in each case.

The temperature of the greenhouse was not allowed to go below 70° F., and only on a few days in May did it rise above 85° F. All experimental rooms except the darkroom were refrigeration rooms, where light intensity, temperature, and humidity were controlled. The darkroom was not refrigerated but for most experiments was maintained at 23° C. or slightly above. Each refrigeration room contained either a 60 or a 75 ampere arc light burning Everready Sunshine car-



bons. This was supplemented by Mazda filament light of between 100 and 200 foot-candles' intensity. Light intensity was controlled by shading the arc lights with thin cloth curtains and by changing the position of the benches on which the plants were placed. Temperatures were kept within 1° C. of that specified. Relative humidity was kept within 80-100 per cent.

In several of the experiments determinations of the effect of temperature, light intensity, and other factors during a single photoperiod were made. In such cases some method must be used to remove, or at least to reduce, the effect of the light which the plants previously received in the greenhouse. It has been shown (2) for *Xanthium* that exposure to repeated short cycles, each consisting of 2-3 hours of darkness followed by 2-3 minutes of medium light intensity, has this effect. Several hours of this treatment following a long bright photoperiod lowers the effectiveness of the photoperiod to the extent that the plants may be exposed to a long dark period and no initiation of flowers will result. In the experiments presented here, except as otherwise specified, a single cycle of this treatment consisted of a 1-hour dark period followed by a  $3 \pm 1$ -minute photoperiod. The light intensity during the photoperiod was 40 foot-candles; the temperature during the entire cycle was 23° C. Exposure to several such consecutive cycles is here designated as short-cycle treatment. The cycles were usually repeated consecutively for a period of 40 hours. In some cases the control units used to test the effectiveness of the short-cycle treatments did not consist entirely of vegetative plants. However, the residual effects from previous photoperiods are lowered sufficiently so that they are probably of little significance.

As has previously been demonstrated, and as is shown in experiment 6, the duration and intensity of the light of the photoperiod immediately following a long dark period have pronounced effect on subsequent flower initiation and development. This factor was therefore controlled as much as possible. Immediately following all long dark periods the plants were placed under uniform light and temperature. Here they were exposed to a photoperiod of at least 8 hours. Following this, the plants were taken out during a period of daylight and placed on the long-day bench of the greenhouse. If, as frequently was the case, the 8-hour photoperiod did not end during daylight, it was extended until 9:00 A.M. or later of the following day, at which time the plants were removed to the greenhouse.

Experiments 5-7 were carried out at Chicago during the summer of 1939. Here the procedure was variable and will be given under each experiment. The recorded stages of floral development do not correspond with that used in experiments 1-4 and 8, but follow that previously used by HAMNER (2).

### Procedure and results

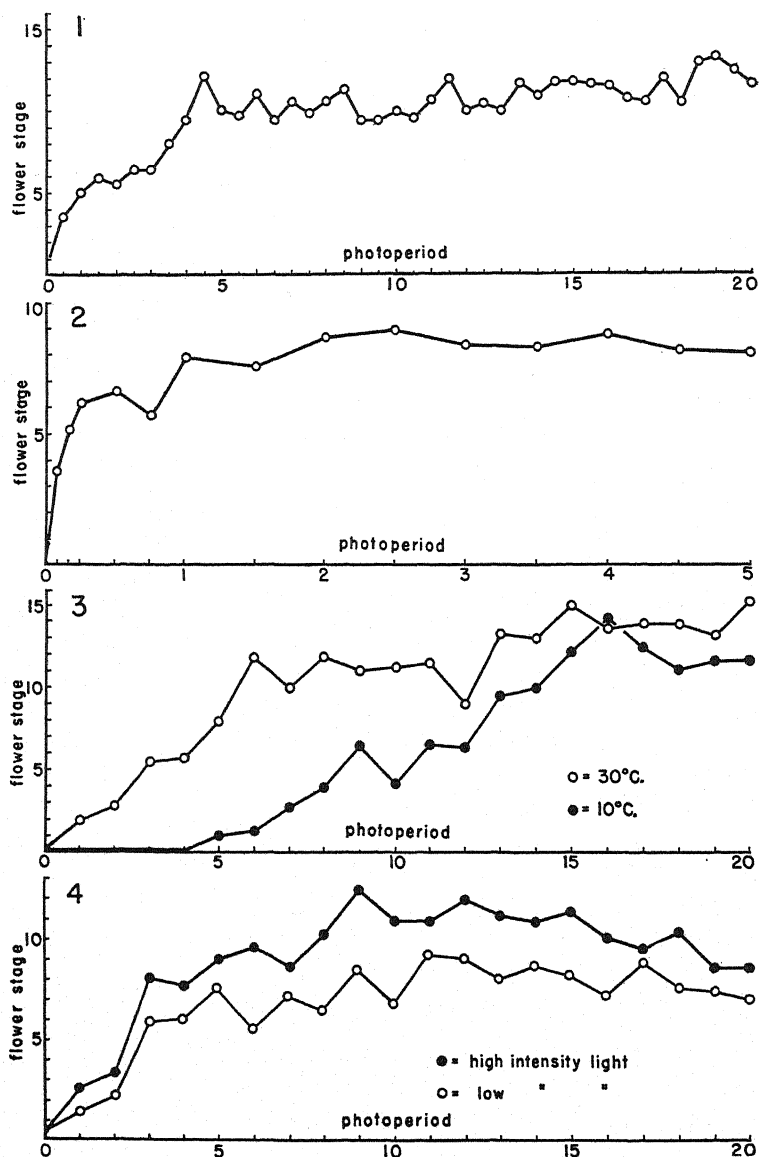
#### EXPERIMENT 1: INITIATION AND DEVELOPMENT OF FLOWERS AFFECTED BY LENGTH OF SINGLE PHOTOPERIOD PRECEDING LONG DARK PERIOD

Plants of *Xanthium* were given an experimental treatment consisting of a single controlled photoinductive cycle, during which induction of flowers might take place. Previous to this controlled cycle, the plants were given a short-cycle treatment. During the single photoinductive cycle all factors remained constant except the length of the photoperiod, which was varied from  $\frac{1}{2}$  to 20 hours. This photoperiod, regardless of length, was followed by a 12-hour dark period.

In accordance with the general outline for experiments given before, 410 plants of a seeding of February 12 were selected from the long-day bench and placed in a control room at noon, March 12. In order to reduce the effect resulting from exposure to light in the greenhouse, the entire lot received a short-cycle treatment lasting 40 hours. Immediately following this, ten of the plants (controls) were placed in the darkroom for 12 hours. The remaining 400 plants were exposed to light of 2000–3000 foot-candles at the leaf surface, with a uniform temperature of 23° C. One-half hour later, and at each  $\frac{1}{2}$ -hour interval thereafter, a unit of ten plants was transferred to the darkroom, 20 hours being required to remove all 400 plants. Thus the units received photoperiods ranging from  $\frac{1}{2}$  to 20 hours. Any given unit remained in the darkroom for 12 hours, after which time it was returned to the arc room for 8 hours or more so that it could be replaced on the long-day bench during daylight. Twenty-two days after all plants had been returned to the bench, they were harvested, the terminal buds examined, and the stage of flowering recorded.

The results of this experiment are shown in figure 1. The length of the variable photoperiod following the short-cycle treatment and preceding the 12-hour dark period is indicated by the abscissa. The average of the stage of flowering of each unit is indicated by the ordinate, which may also be interpreted as indicating the intensity of condition A as affected by the length of the single photoperiod shown on the abscissa. In the lower lefthand corner is indicated the average stage of flowering of the control unit, which received no photoperiod following the short-cycle treatment or preceding the 12-hour dark period. In this unit one plant was flowering and nine were strictly vegetative. Of the other units of the experiment, none contained more than one vegetative plant.

The average of the stage of flowering increases rapidly with the increasing length of the photoperiod up to 4–5 hours. Beyond this, increasing the length of the photoperiod has much less effect on the initiation and development of flower primordia.



FIGS. 1-4.—Effect of varied lengths of photoperiod (single photoinductive cycle) on initiation and development of flower primordia in *Xanthium*: Fig. 1, of medium light intensity; fig. 2, of high light intensity; fig. 3, at two temperatures; fig. 4, at two light intensities.

EXPERIMENT 2: REPETITION OF EXPERIMENT 1, USING PHOTOPERIOD  
OF HIGH LIGHT INTENSITY

This experiment was designed to determine the effect of the length of a single photoperiod preceding a long dark period on the initiation and development of flowers. The procedure differed in several respects from that of experiment 1.

Experiment 1 showed that a single  $\frac{1}{2}$ -hour photoperiod of moderate light intensity preceding a long dark period is sufficient to bring about the initiation of flower primordia. In experiment 2, in which a very high light intensity photoperiod was used, several photoperiods shorter than  $\frac{1}{2}$  hour were given. Another precaution was also taken. The ten control plants in experiment 1 contained one which developed primordia, indicating that there still remained a measurable effect owing to the photoperiod to which the plants had been exposed while on the long-day bench. To minimize this effect still further, the type of short-cycle treatment usually given was considerably modified.

On May 1, 267 uniform plants were selected from a seeding of March 26, which had been growing on the long-day bench, and were placed in a room where they received Mazda filament light of about 5 foot-candles for 18 hours. This treatment was followed by cycles consisting of a 2-hour dark period followed by a photoperiod of  $3 \pm 1$  minutes of 5 foot-candles of Mazda filament light. This type of treatment was continued for 73 hours at a temperature of  $19^{\circ}$ – $20^{\circ}$  C. Immediately following this treatment, one unit of eighteen plants (controls) was taken to a darkroom, avoiding exposure of these plants to light. This unit remained in the darkroom for 12 hours, when it was taken directly to the long-day bench. The remaining plants were divided into units of eighteen plants each. These were given varying lengths of photoperiods by exposure to direct sunlight outside the greenhouse. The light intensity remained above 10,000 foot-candles throughout the exposures; and since these were given during the early part of the day, the temperature rose from  $15^{\circ}$  C. at the beginning to  $22.5^{\circ}$  C. at the conclusion of the longest exposure. The photoperiods varied in length from 5 minutes to 5 hours (fig. 2). At the conclusion of any photoperiod, the unit was placed in a darkroom where it remained for 12 hours. After receiving the dark period, the unit was returned to the long-day bench, where all plants remained until dissected.

The data are given in figure 2. The abscissa and ordinate are similar to those in figure 1, except that the length of some of the photoperiods is different. The stage of flowering of the control unit, which received no photoperiod preceding a 12-hour dark period, is shown in the lower lefthand corner of the graph. All plants of this unit were strictly vegetative. Among the other units represented on the graph, that exposed to a 5-minute photoperiod contained five vegetative plants

out of eighteen, and the unit exposed to a 10-minute photoperiod contained three out of eighteen. No other lots contained more than one.

For a single photoperiod of high light intensity under the conditions of this experiment, the stage of flowering as here measured increased rapidly with increase in the length of the photoperiod up to about 2 hours. Increasing the length beyond 2 hours only slightly affected the stage of flowering of the plants.

EXPERIMENT 3: INITIATION AND DEVELOPMENT OF FLOWER PRIMORDIA  
AFFECTED BY TEMPERATURE DURING SINGLE PHOTOPERIOD  
PRECEDING LONG DARK PERIOD

*Xanthium* which had been grown under long-day conditions was submitted to only one photoinductive cycle, during which induction of the flowering condition might take place. As in experiment 1, preceding and following this treatment the plants were grown on the long-day bench. To reduce the effect of the photoperiods previous to the controlled photoinductive cycle, short cycles were employed. To determine the effect of temperature during the photoperiod, plants were placed in control rooms at two different temperatures, where they received photoperiods varying from 1 to 20 hours.

From plants seeded February 20 and grown under conditions of long day, 410 plants were selected. At noon, March 29, these were placed in a control room and exposed to short-cycle treatment for 40 hours. Immediately following this treatment a unit of ten plants (controls) was placed in the darkroom. The remaining 400 plants were divided into two groups of 200 each, and each group was placed in one of two control rooms, one maintained at 30° C. and one at 10° C. In both rooms the light intensity was 2000-3000 foot-candles at the leaf surface. Beginning the first hour after the plants were placed in the light rooms, units of ten plants were removed hourly from both rooms to the darkroom. Any given unit remained in the darkroom for 12 hours, after which it was returned to a control room maintained at 23° C. and having 2000-3000 foot-candles of light. After 8 hours or more in this control room the units were removed during the day to the long-day bench, where they remained until dissection.

Figure 3 gives the results of this experiment. The stage of flowering of the control unit of nine plants (one died) is shown in the lower lefthand corner of the graph. Two of these nine plants showed the first stages of flowering; the remaining seven were strictly vegetative. Of the other units in which there was more than one vegetative plant, the following list gives the lengths of the photoperiods to which they were exposed and the number of vegetative plants in each case. Among the units at 30° C., the following showed more than one vegetative plant: 1 hour, six vegetative; 2 hours, four vegetative; 3 hours, three vegetative; 4 hours, three vegetative; 5 hours, three vegetative. Among the units at 10° C., the following

contained more than one vegetative plant: 1 hour, nine vegetative; 2 hours, nine vegetative; 3 hours, all vegetative; 4 hours, all vegetative; 5 hours, three vegetative; 6 hours, three vegetative; 7 hours, three vegetative; 10 hours, five vegetative; 12 hours, two vegetative.

At a temperature of 30° C. the response of the stage of flowering to the length of the photoperiod is much like that found in experiment 1; there is a marked rate of increase in the stage of flowering with increase in the length of photoperiod up to about 6 hours. Lengthening photoperiods beyond this increases their effectiveness only slightly. At a temperature of 10° C. a photoperiod shorter than 4–5 hours had little effect so far as initiation and development of flowers were concerned.

The ordinate in figure 3 may also be interpreted as an indicator of the intensity of condition A developed by photoperiods of different lengths and at two different temperatures. In this case the level of condition A rose much more slowly in plants exposed to a photoperiod at 10° C. than in plants exposed to a photoperiod at 30° C. At 10° C., with the light intensity and quality used in this experiment, exposures to light must be 13 hours or more to bring A to the same level reached during a photoperiod of about 6 hours at 30° C. It is evident that, at least for *Xanthium*, the temperature during the photoperiod of a photoinductive cycle has a pronounced effect on subsequent initiation and development of flowers.

#### EXPERIMENT 4: INITIATION AND DEVELOPMENT OF FLOWERS AFFECTED BY INTENSITY OF LIGHT DURING SINGLE PHOTOPERIOD PRECEDING LONG DARK PERIOD

Following the procedure of experiment 3, the selected plants were exposed to a single photoinductive cycle during which induction of flowers might take place. Preceding and following this cycle the plants were grown under conditions of long photoperiod. The plants were divided into three lots, which were placed in separate rooms, each being exposed to a different light intensity during the single controlled photoinductive cycle. In each room the respective lots were exposed to photoperiods ranging from 1 to 20 hours. The final stage of flowering of any given unit is thus a function of the length of the photoperiod and of its light intensity.

On April 22, 610 plants of a seeding of March 13 were selected from the long-day bench of the greenhouse. To reduce the effect of the previous photoperiods, the plants were placed in a control room where they received 40 hours of short cycles. Following this treatment one unit of ten plants (control) was taken immediately to the darkroom, where it remained for 12 hours. The remaining 600 plants were divided into three lots of 200 each, which were distributed to the three arc-light rooms, all of which were kept uniformly at 23° C. In each room the plants were exposed to a different intensity of light at the leaf surface: 115–170 foot-candles in one room, 500–680 in another, and 800–1300 in the third. A unit of

ten plants was removed from each room 1 hour after the plants had been placed therein, and each hour thereafter, 20 hours being required to remove all the plants. Each unit removed was placed in the darkroom and left there for 12 hours, after which it was returned to a control room maintained at 23° C., where it received 2000–3000 foot-candles at the leaf surface. After remaining in this control room for 8 hours or more, the plants were returned to the long-day bench during daylight, where they remained until dissected.

The results of a part of this experiment are given in figure 4. The abscissa indicates the length of the variable photoperiod in hours, and the ordinate, the average of the stage of flowering for the various units of ten plants. Except for a more rapid increase during the first 2 hours of exposure, the stage of floral development of the plants exposed to the photoperiod of highest light intensity was not greatly different from that of those exposed to the medium intensity. For this reason the data for the high intensity are not shown in the graph.

The control unit, the stage of flowering of which is shown at the extreme lower lefthand corner of the graph, contained one flowering and nine vegetative plants. Of the other units containing more than one vegetative plant, the following list gives the length of photoperiod and the number of vegetative plants in each case. At the low intensity there were: 1 hour, eight vegetative; 2 hours, four vegetative; and 6 hours, two vegetative. At the medium intensity there were: 1 hour, three vegetative (nine plants in all); and 2 hours, four vegetative.

The stages of floral development resulting from exposure to these two light intensities are well separated for all photoperiods of the same length; that is, the level of condition A resulting from exposure to the lower intensity shows no tendency to approach the level at the higher intensity, even after prolonged exposure to light.

Figure 4 indicates, as do figures 1 and 3, that the stage of flowering, or the intensity of condition A, increases rapidly with increase in the length of the photoperiod for the first few hours of exposure to light, after which further increase in the photoperiod increases the stage of flowering only slightly. Further, although different light intensities may produce marked differences in the level of A, within wide ranges of light intensity this difference is relatively small.

EXPERIMENT 5: INITIATION AND DEVELOPMENT OF FLOWERS AFFECTED BY  
LENGTH OF SINGLE DARK PERIOD FOLLOWING LONG  
PHOTOPERIOD OF HIGH LIGHT INTENSITY

*Xanthium* may be kept indefinitely vegetative if exposed to photoperiods of 18–20 hours with intervening dark periods of 6–4 hours. If one of these cycles is modified by increasing the length of the dark period beyond 8½–9 hours, the plants are induced to flower even though subsequently they may be returned to conditions of long photoperiod or continuous light.

For dark periods around  $23^{\circ}$  C. the minimum length of the dark period necessary to induce flowering is  $8\frac{1}{2}$ –9 hours. It has been shown (experiment 8) that several photoinductive cycles with long dark periods are more effective in the induction of flower primordia—and especially in the development of flowers—than a single cycle. That the effectiveness of a single long dark period might be augmented by increasing it beyond the  $8\frac{1}{2}$ –9 hours' minimum length necessary to induce flowering is a possibility.

This experiment was carried out at Chicago during the summer of 1939. The plants were grown on the long-day bench from June 2 until June 30. Following a bright day, units of ten plants each were taken from the long-day bench and given dark periods of various lengths, as indicated in figure 5. Following this dark period the units were exposed to either of two conditions: part of them were returned to

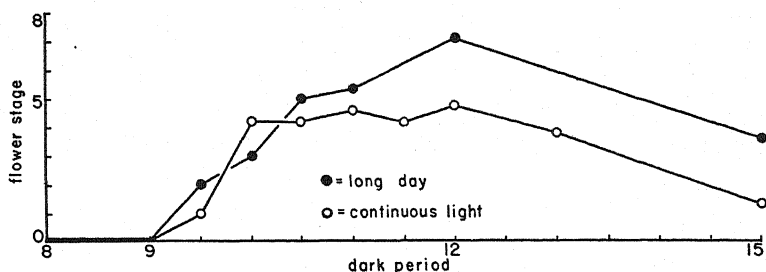


FIG. 5.—Effect of length of single dark period and subsequent photoperiodic treatment on initiation and development of flower primordia in *Xanthium*. Plants received single photoinductive cycle.

the long-day bench and part were placed under continuous light (daylight extended by Mazda filament light of about 100 foot-candles from sundown to sunrise). As indicated by unpublished experiments, and as found by HAMNER (3), long photoperiods and short dark periods following flower induction are more favorable to flower development than is continuous light.

The results of this treatment are shown in figure 5. The abscissa indicates the length of the single long dark period to which the plants were exposed; the ordinate indicates the relative amount of initiation and floral development. One line in the graph represents those plants returned to long-day conditions to develop; the other line, those which remained on continuous light. As noted under the section on methods, the scale for stage of flowering used here differs from that in experiments 1–4 and 8.

Each experimental unit consisted of ten plants. Under the conditions of this experiment all plants given dark periods of  $8\frac{1}{2}$ –9 hours remained strictly vegetative. Under usual experimental conditions, at least some plants receiving a dark period of this length would flower. This and the relatively large numbers of vege-



tative plants listed below probably resulted from the removal of the plants from the dark period into early morning light of rather low intensity (see experiment 6). Among those units returned to long-day conditions after treatment, the following contained more than one vegetative plant: 9.5 hours, six vegetative; 10 hours, four vegetative; 15 hours, four vegetative. Among those lots returned to continuous light after treatment, the following contained more than one vegetative plant: 9.5 hours, seven vegetative; 10 hours, two vegetative; 11 hours, three vegetative; 11.5 hours, three vegetative; 13 hours, two vegetative; 15 hours, three vegetative.

This experiment has been repeated twice, employing dark periods up to 50 hours and allowing the plants to develop on long photoperiods. Both repetitions indicate a maximum effective length of 12-15 hours for the dark period.

EXPERIMENT 6: INITIATION AND DEVELOPMENT OF FLOWERS AFFECTED BY  
PHOTOPERIODS OF LOW LIGHT INTENSITY IMMEDIATELY  
PRECEDING AND FOLLOWING LONG DARK PERIOD

For *Xanthium* plants kept continuously vegetative by growing under conditions of long day, the length of the dark period is the main factor limiting the induction

TABLE 1  
EFFECT OF LOW LIGHT INTENSITY ON INITIATION AND DEVELOPMENT OF FLOWERS

TREATMENT		AVERAGE STAGE OF FLOWERING	NO. OF PLANTS	
PHOTOPERIOD PRECEDING 12-HOUR DARK PERIOD	PHOTOPERIOD FOLLOWING 12-HOUR DARK PERIOD		VEG.	FL.
12 hours of 200 f.c. Mazda filament light	{ 12 hours of 200 f.c. Mazda filament light. ....	0.0	20	0
	{ 12 hours of 1200 f.c. arc light. ....	0.3	18	2
12 hours of 1200 f.c. arc light	{ 12 hours of 200 f.c. Mazda filament light. ....	0.15	16	4
	{ 12 hours of 1200 f.c. arc light. ....	3.5	0	20

of the flowering condition. Under usual growing conditions the lengthening of a single dark period beyond  $8\frac{1}{2}$ -9 hours will bring about initiation. With more careful consideration of the rate of development of primordia, other factors affecting the relative strength of a photoinductive cycle are found in addition to the length of the photoperiod or dark period. A number of these have been indicated in the previous experiments. The intensity of the light of the photoperiod immediately preceding and/or following a long dark period is of considerable importance.

Selected plants were given a single dark period of 12 hours preceded and followed by 12-hour photoperiods of controlled light intensity. On October 7 eighty plants from the long-day bench were divided into two lots of forty each, one lot receiving Mazda filament light at an intensity of 200 foot-candles and the other re-

ceiving arc light of about 1200 foot-candles, for 12 hours; then each lot received a 12-hour dark period. At the end of this dark period, half the plants in each of the two treatments were returned to the light of 200 foot-candles for 12 hours, and the remaining half were placed under the light of 1200 foot-candles. At the end of this treatment the four groups were returned to the long-day bench, where they remained until harvested for dissection, 27 days after the start of the experiment. The method of measuring the flower stage in the following data is different from that used in experiments 1-4 and 8. The nature of the treatments and the data are summarized in table 1.

EXPERIMENT 7: INITIATION AND DEVELOPMENT OF FLOWERS BY PHOTO-INDUCTIVE CYCLES WITH LOW LIGHT INTENSITY PHOTOPERIOD

Experiment 6 indicated that decreasing the intensity of light preceding and following a long dark period markedly decreased the effectiveness of that dark

TABLE 2  
EFFECT OF PHOTOPERIODS OF LOW LIGHT INTENSITY  
ON INITIATION AND DEVELOPMENT OF FLOWERS

NO. OF PHOTO- PERIODS, EACH SEPARATED BY 12-HOUR DARK PERIOD	LIGHT INTENSITY IN F.C. DURING PHOTOPERIOD	AVERAGE STAGE OF FLOWERING	NO. OF PLANTS	
			VEG.	FL.
2	18.....	0.0	9	0
	50.....	0.0	10	0
	100.....	0.0	10	0
11	18.....	1.4	3	4
	50.....	1.6	2	8
	100.....	2.3	1	6

period in inducing flower primordia. However, a photoperiod of very low intensity may be effective in bringing about induction of flowers if the photoinductive cycles are repeated several times.

At 8:00 A.M., October 6, sixty plants were removed from the long-day bench, and two units of ten plants each were placed under each of three intensities of Mazda filament light—18, 50, and 100 foot-candles. After a 12-hour exposure, all plants received a 12-hour dark period. This dark period was followed by an exposure to 12 hours of light of the same intensity to which the unit was first exposed. Half the lots were then returned to the long-day bench, where they remained until dissected, while the remaining half received nine more of these cycles before being returned to the bench. All plants were dissected November 4. The experimental procedure and results are presented in table 2.

EXPERIMENT 8: EFFECT OF NUMBER OF PHOTOINDUCTIVE CYCLES ON RATE  
OF DEVELOPMENT OF FLOWERS AND FRUITS GROWN UNDER  
CONDITIONS OF LONG DAY

Table 3 shows in a general way the effect which the number of induction periods has on the rate of development of flower structures and fruits. The first visible evidence of inflorescence initiation appears 2-3 days following a single induction period of the type given here. For this reason the effect of the number of inductive

TABLE 3

NUMBER OF CYCLES EACH CONSISTING OF A 12-HOUR PHOTOPERIOD FOLLOWED BY A 12-HOUR DARK PERIOD. EXPERIMENT STARTED THE MORNING OF APRIL 2. ALL FIGURES WITHOUT QUALIFYING TERMS REFER TO STAGE OF FLOWERING

DATE EXAMINED	CYCLES				CONTINUOUSLY REPEATED
	1	2	4	8	
April 5.....	1.0	1.0	1.0	.....	.....
April 8.....	2.6	3.6	4.6	4.8	.....
April 11.....	3.3	9.1	12.3	11.6	.....
April 14.....	4.4	12.3	17.4	18.7	.....
April 17.....	7.4	13.4	23.3	33.3	.....
April 22.....	9.4	13.8	24.0	43.8	.....
April 24.....	.....	18.0	30.5	Pollen first shed	Pollen being shed
April 29.....	11.4	18.8	44.8	Bur length 14.2 mm.	Bur length 20.5 mm.
May 6.....	.....	.....	Pollen first shed	.....	.....
May 8.....	17.8	28.3	.....	.....	.....
June 4.....	Pollen being shed	Bur length 12.2 mm.	Bur length 33.3 mm.	Burs nearly mature, becoming brown	Burs completely mature, length 22.4 mm.; plants dead

cycles is much less evident in earlier stages of development of inflorescence and flower primordia than in the later stages. The later development proceeds much more rapidly in plants which received several short-day treatments than in plants which received only one or two such treatments.

Plants were selected April 2 from a group seeded on the long-day bench February 20. The cycles used for flower induction consisted of a 12-hour photoperiod of natural daylight in the greenhouse (6:00 A.M. to 6:00 P.M.) followed by a 12-hour dark period. The various lots received 1, 2, 4, 8, and continuously repeated cycles. All lots except the last were returned to the long-day bench for development after treatment. The stage of flowering is measured on the same scale as that used for experiments 1-4.

By June 4, 64 days after induction began, the plants on continuous short day were completely mature and had died. At the other extreme were those plants which had received only one long night and were just beginning to shed pollen. Plants developing on continuous short days bore fruit which differed markedly from that of plants induced with a few short days and then allowed to develop on long days. The burs of the former are much smaller, bear only a few spines (in comparison with those on long day), and frequently the thin involucre is broken, allowing the ovary to protrude for half its length.

### Discussion

The effect of temperature during the dark period may be compared with that during the photoperiod. LONG (4) has shown that lowering the temperature increases the critical length of the dark period. The temperature during the dark period is also of importance in flower induction in Biloxi soybeans (5). It has been previously found with *Xanthium* that temperature had little effect on the photoperiodic reaction (4). Experiment 3, however, shows definite temperature effect. For short photoperiods this effect was pronounced, but it decreased as the photoperiods increased in length. Previous workers, when considering the relative effects of temperature on the dark period and the photoperiod, have used dark periods near the critical length but photoperiods far in excess of the minimum length. This probably explains the resultant relatively low coefficient of temperature for the reactions of the photoperiod. In Biloxi soybean the effect of temperature was found to be slight but significant (5).

In comparing the results of these experiments with other data, especially from other species, it must be kept in mind that the flowering responses in experiments 1-6 depended upon variable factors introduced into a single photoinductive cycle. The plants were exposed to only one cycle during which flower induction could have taken place. Many short-day plants, such as Biloxi soybean, require several cycles of treatment for such induction. LONG (4) has shown that when several cycles are given, the time elapsing between consecutive cycles is of great importance. This spacing may determine in part the most effective length of photoperiod or dark period for induction of flowers, as has been suggested by HAMNER (2). Thus in experiment 5 the optimum length for the dark period was found to be 12-15 hours, although this would probably be decreased if several cycles were given instead of one, since long dark periods space the photoinductive cycles farther apart. When several photoinductive cycles are given, this effect must be considered.

The importance of the light period immediately following a dark period has been neglected in most work with *Xanthium*. Experiment 6 indicates that the photoperiod following a long dark period—as well as that preceding it—may be impor-

tant. When plants are exposed to several consecutive photoinductive cycles, a photoperiod both follows and precedes a dark period. The role of the photoperiod under these conditions may be somewhat different from a single-cycle experiment, where the test photoperiod only precedes a dark period. Experimental work on the effect of light immediately following a dark period is needed.

In the preceding experiments reference has been made to condition A, which has been defined as "the changes or conditions which arise owing to exposure to light" (2). Such changes or conditions are those which develop on exposure to high light intensity at moderate temperatures. Within certain limits the intensity of A increases with increasing exposure to light. In addition to its function of establishing condition A, but probably distinct from it, light interferes with the reactions which occur during the dark period following a photoperiod. Here continuous light of very low intensity or periods of moderate intensity and of very short duration are effective. In *Xanthium*, 1 minute of light in the middle of a 9-hour dark period will nullify the effect of the dark period so far as the initiation of flowers is concerned. In Biloxi soybean, BORTHWICK and PARKER (1) have demonstrated that Mazda light of 0.6 foot-candles, given for the first 8 hours of a 16-hour dark period, will prevent flower induction.

In *Xanthium* and Biloxi soybean condition A is developed most rapidly under light of high intensity. On the other hand, development of condition B depends on the exclusion of light, 0.6 foot-candles being sufficient to inhibit this condition in the soybean. There is nothing to indicate that the reactions brought about by light of high intensity (the establishment of condition A) are the same as those which occur when light interferes with the reactions taking place in the dark.

If two distinct reactions or groups of reactions are present, the recognition of this fact is of some importance. WITHROW and BIEBEL (6), using *salvia*, a short-day plant, found that flowering plants would return to the vegetative state if placed on long photoperiods. Giving these plants a short photoperiod of natural daylight and extending this with additional low light intensity also caused the plants to revert to the vegetative condition; that is, the short photoperiod supplemented by low light intensity brought about a response characteristic of long photoperiods. At low intensities red radiation was found to be more effective as supplementary light than other portions of the spectrum used. They conclude that, "under the conditions of the experiment, red radiation is most effective in producing the photoperiodic response both in long and short day plants." It would seem that, with respect to short-day plants, their experiments dealt with the second mentioned response to light rather than with the establishment of condition A, and indicate that red radiation is most effective in interrupting the dark period—in interfering with condition B.

### Summary

1. Induction of flowers may take place in vegetative plants of *Xanthium pennsylvanicum* when subjected to a single controlled photoinductive cycle. This flowering response is recorded on a more extensive scale than has previously been used. The measurement is based on the size of the apical inflorescence.

2. The effect of the length of the photoperiod given during this single cycle is related both to its temperature and to its light intensity. In general, the initiation of flower primordia and their rate of development increase with the increasing length of the photoperiod up to a rather definite point, beyond which further lengthening of the photoperiod has little effect.

3. By increasing the light intensity, the effectiveness of the photoperiod of the single photoinductive cycle is increased, at least for large differences of light intensity. This response seems independent of the length of the photoperiod, being evident even after 20 hours. Photoperiods of as low as 18 foot-candles may bring about induction if repeated consecutively several times.

4. The temperature during the photoperiod has a pronounced effect on the resultant stage of flowering. At 10° C. a single photoperiod of 2000–3000 foot-candles must be in excess of 4–5 hours to cause flowering. At 30° C. a photoperiod of similar intensity but only  $\frac{1}{2}$  hour in length may cause initiation. As the photoperiod increases in length, the effect of the temperature diminishes.

5. In plants induced to flower by increasing the length of a single dark period beyond 8½–9 hours, the maximum stage of flowering is brought about by dark periods of 12–15 hours.

6. The photoperiod following a long dark period—as well as that preceding it—has a pronounced effect on the initiation and development of flowers.

7. Data are given showing the influence of the number of photoinductive cycles to which plants are exposed on the rate of development of flower structures.

8. Some of the data of these experiments are interpreted on the basis of HAMNER's suggestion that the induction of certain short-day plants may be represented by  $A, B \rightarrow C$ .

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# EFFECT OF BILE SALTS AND OLEATES ON THE STRUCTURAL VISCOSITY OF PROTOPLASM<sup>1</sup>

ROBERT M. MUIR

(WITH THREE FIGURES)

## Introduction

As a result of facts derived from many investigations upon the nature of protoplasm, BAITSELL (2), FREY-WYSSLING (4), NEEDHAM (5), PETERS (6), NORTEN (7), and others have concluded that protoplasm has a definite structure. According to SEIFRIZ (10), this continuity of structure may be regarded as the determinant of the living as opposed to the nonliving. With further knowledge concerning naturally occurring substances, the proteins in particular, the nature of this structure becomes more and more clarified.

The basic units of protoplasmic structure are conceived as being the fibrous or threadlike molecules of proteins, which through the linkages of their side chains form a network structure to which NEEDHAM (5) applied the term "cytoskeleton." PETERS (6) has elaborated this concept by distinguishing three parts: (a) the surface proteins; (b) the cytoplasmic proteins (with only slight chemical differences from the surface proteins but more important differences of orientation); and (c) the nuclear proteins.

Fats and fatlike substances are regarded as indispensable constituents of living tissue, with functions based upon their solubility relationships and physical as well as chemical properties. OSBORNE (9) has concluded that a chemical union, unstable toward alcohol, exists between the protein and the phosphatide of lecithovitellin of egg yolk. Experiments by MÂCHEBOEUF (9) and by SØRENSEN (9) indicate that the linkage between the lipid and the protein is probably a residual or nonpolar valency linkage. It is possible that the lipids, in part, function as a cement to connect the protein molecules into a network or cytoskeleton, and have a fundamental role in dissociation reactions (12).

FREY-WYSSLING (4) has suggested the incorporation of phosphatides into the protein reticulum through heteropolar cohesion bonds, or secondary valency bonds, between hydrophilic side chains ( $\text{—OH} \dots \text{HO—}$ ), and through homopolar cohesion bonds between lipophilic side chains ( $\text{—CH}_3 \text{ H}_3\text{C—}$ ). Likewise the fats would be associated with the protein chains directly or through phosphatide molecules by means of the homopolar cohesion bond.

<sup>1</sup> Contributions from the Department of Botany and the Rocky Mountain Herbarium of the University of Wyoming, no. 182.



In view of such a protoplasmic structure, based upon a protein network in which fats and lipoids are incorporated, experiments were performed with substances such as sodium glycocholate, sodium taurocholate, sodium oleate, and magnesium oleate (which are known to affect fats and lipoids) to determine their effects upon the structural viscosity of protoplasm.

### Method

The method of measurement of changes in structural viscosity is based upon the equation:  $V = k(c - c_0)$ , derived by NORTHERN (7), which approximately governs the rate with which the chloroplasts in cells of *Spirogyra* move in response to different centrifugal accelerations. In the equation,  $V$  is the velocity of chloroplastic movement,  $k$  a constant,  $c$  the centrifugal acceleration, and  $c_0$  is the initial centrifugal acceleration at which—or below which—the chloroplasts will not move regardless of the length of time the acceleration is allowed to act.

In general, the acceleration that would displace the chloroplasts in only 5–20 per cent of the filaments was used. The different accelerations usually employed in determining this particular one were  $1062 \times$  gravity,  $680 \times$  gravity,  $382 \times$  gravity,  $245 \times$  gravity, and  $170 \times$  gravity. If the acceleration were increased to the next higher value, 50 per cent or more of the filaments would have the chloroplasts displaced.

The  $c_0$  value is not uniform for all *Spirogyra* filaments in a sample group; therefore, when a group of filaments is centrifuged with an acceleration of, for example,  $680 \times$  gravity, the chloroplasts will be displaced in the cells of those filaments whose  $c_0$  is less than  $680 \times$  gravity and will not be displaced in cells with a  $c_0$  value equal to or greater than  $680 \times$  gravity. Considering the spread of  $c_0$  values for the different filaments, any treatment which decreases the structural viscosity (lowers the  $c_0$  value) will cause an increase in the number of filaments with the chloroplasts displaced when centrifuged with an acceleration of  $680 \times$  gravity. Likewise any treatment which increases the structural viscosity (raises the  $c_0$  value) will cause a decrease in the number of filaments with the chloroplasts displaced.

Filaments of *Spirogyra* were immersed in tapwater solutions of  $0.001M$  sodium glycocholate,  $0.001M$  sodium taurocholate,  $0.001M$  sodium oleate, and  $0.0006M$  magnesium oleate. All solutions had a pH value of 7.5. After the filaments had been in the solution for the desired time, a group of them was removed, placed between strips of cotton soaked in the solution, and centrifuged. A group of untreated filaments (control) was centrifuged each time with the group of treated filaments. After a certain period of immersion, during which the relative viscosity had been determined at intervals for the experimental groups, the remaining filaments were transferred to tapwater and groups of them were centrifuged at intervals to note recovery from the effects of immersion in the solution. Following

centrifugation, the percentages of filaments having cells with the chloroplasts displaced were determined. For each experimental group between 350 and 450 filaments were examined.

### Data

In recording the data, the percentage of untreated filaments with displaced chloroplasts was subtracted from the percentage of treated filaments with the chloroplasts displaced. (The two groups had been centrifuged synchronously.) Thus the figure recorded is the percentage of filaments in which the cells had the chloroplasts displaced as a result of the treatment.

TABLE 1  
EFFECTS OF BILE SALTS UPON PROTOPLASMIC VISCOSITY

TIME OF IMMERSION (MINUTES)	PERCENTAGE FILAMENTS WITH CHLOROPLASTS DISPLACED	
	SODIUM TAUROCHOLATE	SODIUM GLYCOCHOLATE
5.....	7	0
20.....	26	5
40.....	35	23
60.....	47	28
90.....	30	34
Time in solution before removal to water (minutes).....	100	70
Minutes in water:		
30.....	17	0
60.....	.....	0
90.....	0	.....

The results obtained for sodium taurocholate and sodium glycocholate are recorded in table 1. For the sodium taurocholate experiment an acceleration of  $245\times$  gravity was used for a period of 140 seconds; in the control groups the average percentage of filaments with the chloroplasts displaced was 14 per cent. For the sodium glycocholate an acceleration of  $382\times$  gravity was used for a period of 90 seconds; the average percentage of displacement in the control groups was 18 per cent. The taurocholate caused a somewhat greater decrease in structural viscosity than did the glycocholate: the maximum obtained for the former was 47 per cent as compared with a maximum of 34 per cent for the glycocholate. The glycocholate also required a longer time in which to bring about the decrease. In the taurocholate experiment the increase in structural viscosity after 90 minutes' immersion, as compared with the viscosity at 60 minutes, indicates a feature of its effect which is more exactly shown in the following oleate experiments (figs. 1-3).

In the oleate experiments the decrease in structural viscosity is not a cumulative effect but a fluctuating one: a decrease is followed by an increase which in turn is followed by another decrease, etc. The filaments recovered completely from the effect of the glycocholate 30 minutes after removal to tapwater and likewise were well on the way to a normal viscosity value after a 30-minute period in tapwater following treatment with the taurocholate.

The effect of sodium oleate during the first 6 minutes of immersion is recorded graphically in figure 2 (solid line); the effect during 135 minutes is recorded in figure 1. In the latter figure the solid line represents the effect of immersion in the oleate solution, and the broken line shows the trend of recovery when the filaments were removed to water after 135 minutes of immersion. The decrease in

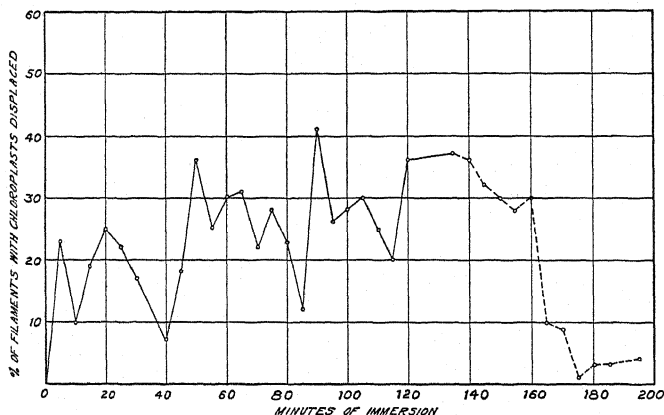
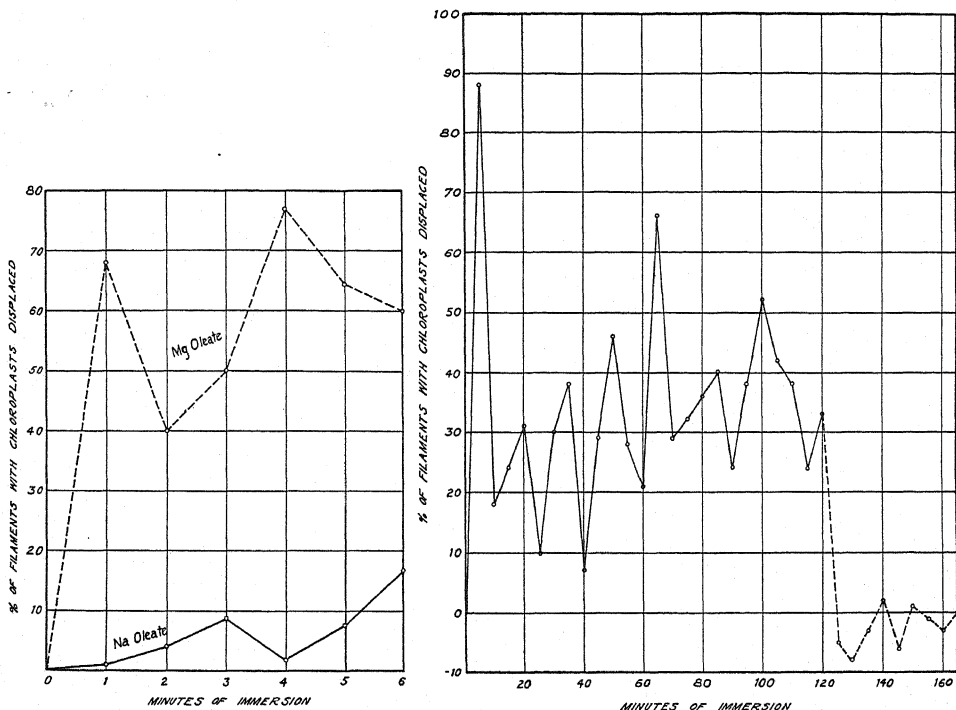


FIG. 1.—Effect of 0.001*M* sodium oleate solution upon structural viscosity of protoplasm in *Spirogyra* filaments as shown by centrifugation (solid line) and effect of removal to water (broken line).

structural viscosity was not pronounced during the first 6 minutes (maximum 17 per cent), and fluctuations were only slight. After a longer period of immersion (fig. 1) the fluctuations assumed major proportions and the decrease in structural viscosity became very significant (maximum 41 per cent). There was only partial recovery after 25 minutes in water, but after 30 minutes recovery was nearly complete. For this experiment the acceleration was  $680\times$  gravity applied for 50 seconds (excepting the seventh, eighth, and ninth recovery determinations for which it was  $382\times$  gravity applied for 90 seconds, and the tenth and eleventh for which it was  $245\times$  gravity applied for 140 seconds). The average percentage of chloroplast displacement in the control groups of filaments was 7 per cent for immersion determinations and 33 per cent for recovery determinations. The magnitude of the latter value is caused by the tendency of the  $c_0$  value to change in response to variations in the intensity of illumination during such long periods of time as were employed in this and the following experiment.

The effect of the magnesium oleate is recorded graphically in figure 2 (broken line) and in figure 3. In the experiment for figure 3 the filaments were removed to water after 120 minutes' immersion. Although the magnesium oleate solution was only  $0.0006M$  instead of  $0.001M$ , and the acceleration was lower than that used in the sodium oleate experiment, the magnesium oleate had great initial effect (maxi-



FIGS. 2, 3.—Fig. 2 (left), effects of  $0.001M$  sodium oleate solution (solid line) and  $0.0006M$  magnesium oleate solution (broken line) upon structural viscosity of protoplasm in *Spirogyra* filaments as shown by centrifugation. Fig. 3 (right), effect of  $0.0006M$  magnesium oleate solution upon structural viscosity of protoplasm in filaments as shown by centrifugation (solid line) and effect of removal to water (broken line).

mum 88 per cent), with pronounced fluctuations. These fluctuations also occurred during the longer period of immersion. The effect of the magnesium oleate in decreasing the structural viscosity did not exceed the effect of the sodium oleate as greatly in the longer period of immersion (maximum 66 per cent) as it did in the initial period. An important difference is also shown in the immediate recovery of the filaments from the effect of the magnesium oleate. When placed in water, the structural viscosity of the treated filaments within 5 minutes became slightly greater than that of the untreated (minimum -8 per cent). For this experiment the

acceleration was  $382\times$  gravity applied for 90 seconds in all determinations. The average percentage of chloroplast displacement in the control groups of filaments was 20 per cent for immersion determinations and 32 per cent for recovery determinations.

### Discussion

The decrease in structural viscosity brought about by the bile salts and the oleates is interpreted as the result of a loosening of the protein cytoskeleton, probably through their effects upon linkages concerning fats and fatlike substances. This may occur in a way analogous to that which STEINHARDT (11) used to explain the dissociation of horse hemoglobin by amides. He proposed that binding forces between parallel polypeptide chains which arise between  $\text{—N—}$  and  $\text{H}$

$\text{—C—}$  groups may be weakened or destroyed as a result of a similar association of  $\text{O}$

the introduced amides with the elements of the grid at the peptide bonds in competition with peptide bonds of adjacent chains. In a similar way substances such as the bile salts and oleates may weaken or destroy the homopolar cohesion bonds between lipophilic side chains described by FREY-WYSSLING through their association with the lipophilic side chains.

The possible effects of bile salts and oleates upon valence linkages—of the nature of those between lipids and proteins as proposed by OSBORNE (9), MACHEBOEUF (9), and SØRENSEN (9)—would seem to be insufficient to account for the ultimate decrease in structural viscosity as observed.

ANSON (1) has found that detergents and bile salts denature ordinary proteins such as hemoglobin and egg albumin, and he considers that, since the detergents and bile salts differ widely in chemical composition but all have one property in common, their hydrophobic-hydrophilic polar character, it is this latter character upon which their reactions with proteins as well as their physiological reactions depend. BURKE (3) has found that the dissociation and denaturation processes in urea solutions of amandin and excelsin are closely correlated, if not one and the same process. The effects of the bile salts upon the protoplasmic proteins, as observed in this investigation, indicate that, although a dissociation occurs, it is not accompanied by denaturation, because there are fluctuations in structural viscosity and because the structural viscosity returns to the normal value when the filaments are transferred to water.

The fluctuations in structural viscosity are interpreted as the result of reassociations following the weakening or destruction of the linkages between the polypeptide chains brought about by the effects of the bile salts and oleates. These fluctuations in structural viscosity do occur, and their interpretation as one of dissociation and reassociation is compatible with the views of SØRENSEN and others

(9) concerning soluble proteins. NORTEN (8) has discussed such variation in protoplasmic viscosity.

Magnesium oleate has a much greater effect than has sodium oleate during the first 6 minutes of immersion (fig. 2). The difference in effect, although not so pronounced, continues during the longer period of immersion (figs. 1, 3). This difference might be explained as the result of a greater attraction between the fats and fatlike substances and the magnesium oleate. The fact that recovery from the magnesium compound's effect was immediate, however, whereas in the case of the sodium compound recovery occurred only after a period of time, makes such an explanation inadequate. The difference in effect could also be considered as a result of the control exercised by the cell membrane. The magnesium oleate would pass through a lipoidal membrane more rapidly than would sodium oleate, but it is doubtful whether its speed of entrance and departure would be great enough to account for the initial effect and the immediate recovery. It will be recalled that 68 per cent of the filaments had the chloroplasts displaced when centrifuged after 1 minute of immersion in the magnesium oleate solution and that 5 minutes after removal to tapwater the viscosity had increased to a value slightly higher than that of the untreated filaments.

The best explanation would seem to be based upon the distinction of "surface proteins" and the continuity between them and the other proteins of the cell. In the theory proposed by PETERS (6), a local change solely in the surface proteins (acceptors)—insufficient to cause permanent upset—could produce a decrease in protoplasmic viscosity by causing temporary dissolution of bonds which then "snap" back into place. The action of magnesium oleate and sodium oleate might then be postulated as consisting of two phases: (a) that concerned with the surface proteins; and (b) that concerned with the cytoplasmic proteins after the substance has entered the cell. The action upon the surface proteins would be of major importance during the initial period of immersion, and if the substance has a pronounced effect upon the surface proteins, the removal of the filaments to water would have an immediate consequence. During longer periods of immersion, the action upon the cytoplasmic proteins of the substance that has entered the cell would be of considerable importance. The action upon surface proteins and the action upon cytoplasmic proteins may then be additive in their effect. The major portion of the effect of the magnesium oleate would result from its action on the surface proteins, whereas the major portion of the effect of the sodium oleate would result from its action on the cytoplasmic proteins.

### Summary

1. Groups of *Spirogyra* filaments were immersed in 0.001*M* tapwater solutions of sodium taurocholate, sodium glycocholate, and sodium oleate, and a 0.0006*M*

solution of magnesium oleate. At intervals, sample lots of filaments were centrifuged in their respective solutions. A control group of filaments from tapwater was always centrifuged with the group of treated filaments. An increase or decrease in the percentage of filaments having cells with the chloroplasts displaced indicated that the viscosity of the protoplasm had been decreased or increased, respectively.

2. All four substances decreased the structural viscosity. The decrease became more and more pronounced the longer the period of immersion in the solutions of the bile salts, except for an increase in structural viscosity at the end of 90 minutes' immersion in the sodium taurocholate solution. The oleates gave pronounced decreases in structural viscosity, followed by increases, which in turn were followed by decreases, etc. The effect of magnesium oleate was greater than that of sodium oleate.

3. As determined at 1-minute intervals during the first 6 minutes of immersion, the magnesium oleate produced a very large initial decrease in structural viscosity with major fluctuations, whereas the sodium oleate had a relatively small initial effect with only slight fluctuations.

4. After a period of immersion, the filaments were removed to tapwater and recoveries from the effects of the substances observed. The recovery from the effect of the sodium glycocholate was entirely completed, and the recovery from sodium taurocholate was half completed within 30 minutes. Recovery was completed within 5 minutes for filaments immersed in magnesium oleate solution but was not completed until 30 minutes in the case of sodium oleate.

5. The decrease in structural viscosity brought about by these substances may be the result of a weakening or destruction of bonds such as those existing between the lipophilic side chains of protein molecules. This is followed by reassociation, increasing the structural viscosity.

6. The rapidity and magnitude of the effect of the magnesium oleate, as compared with that of the sodium oleate, may be explained as the result of a greater effect of the former upon surface proteins and a restriction of the latter's effect more to the cytoplasmic proteins.

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# EFFECT OF INDOLEACETIC ACID ON THIN SECTIONS AND DETACHED SEGMENTS OF THE SECOND INTERNODE OF THE BEAN<sup>1</sup>

CONTRIBUTIONS FROM THE HULL BOTANICAL LABORATORY 518

J. M. BEAL

(WITH TWO FIGURES)

## Introduction

The following experiments concern the effects of indoleacetic acid on detached segments and thin sections of the second internode of the Red Kidney bean (*Phaseolus vulgaris*) grown on WHITE's nutrient solution. The nutrient solution was made up according to WHITE's 1939 formula (2) and solidified to a soft gel by the addition of 0.5 per cent agar.

## Methods and results

The experimental plants were grown in 6-inch pots containing garden soil and were kept under ordinary greenhouse conditions until ready for use. When the second internode had attained a length of 1-2 inches and the leaflets of the first compound leaf were beginning to expand, the stem was cut squarely across about 1 mm. below the base of the petiole of the compound leaf and immediately treated in one of the ways to be described, except for the one experiment in which the second internode was laterally treated.

SERIES 1.—After decapitation, a single application of 0.5 per cent indoleacetic acid in lanolin was spread evenly over the entire cut surface of the shoots. These were left on the plants and kept in ordinary daylight. Preliminary tests showed that the responses of treated stems were apparently as rapid and the histological changes were the same when an indoleacetic acid-lanolin mixture of this concentration was used as when a stronger mixture (such as 3 per cent) was applied, and that there was a marked reduction in injury to the cells at and near the surface of application with the weaker mixture. Only the weaker concentration was used, therefore, in the experiments involving the indoleacetic acid-lanolin mixture.

Following decapitation and application of the mixture, the same sequence of changes was observed in the treated stems in approximately the same time intervals as has been reported previously (1). Both gross aspects and histological responses parallel these descriptions very closely.

<sup>1</sup> This work was aided in part by a grant from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

Transections 0.5 mm. or less thick were made from the apical swellings of stems, treated as described, 24 hours or more after treatment and were then placed on WHITE's solidified nutrient. Before placing them on the nutrient, some were washed quickly in 95 per cent alcohol, some were dipped into a 1-1000 solution of  $\text{Hg}(\text{Cl})_2$  or a 2 per cent solution of  $\text{Ca}(\text{OCl})_2$  followed by rinsing in sterile distilled water, and some were placed immediately on the nutrient with no attempt at disinfection. There was little difference in the behavior of any of the segments, although there was somewhat greater fungus contamination when not sterilized.

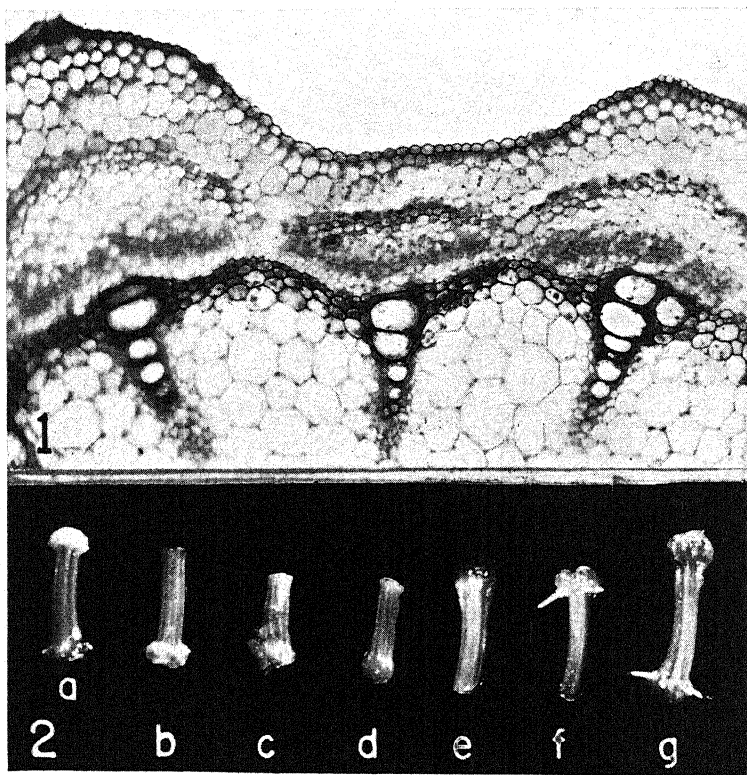
The responses of these sections were compared with those of comparable sections of second internodes treated with lanolin only and also with sections of freshly cut untreated stems which were also placed on the nutrient. In no case do the sections from the second internode not treated with indoleacetic acid proliferate noticeably, although they remain alive on the nutrient for several days. The sections from the swellings induced by the indoleacetic acid, made 24 hours or more after treatment, proliferate freely in the region outside the xylem cylinder, and in 2-4 days the proliferated tissues pile up to form a thick ruffled mass about the xylem. Roots frequently develop from the tissues outside the xylem cylinder and often attain a length of 5 mm. or more. The xylem ray tissues appear to be involved, but no proliferation of the pith was observed.

Pieces of tissue about 1 mm. square were taken—24 hours or more after treatment—from some of the stem swellings outside the xylem cylinder and placed on nutrient. These proliferate noticeably and often produce one or two roots each. It is probable that root primordia were present in some of the squares and that they continue development on the medium.

SERIES 2.—Other stems, without decapitation, were treated laterally by smearing the mixture in a narrow streak longitudinally along one side of the shoot. Within 1-1½ hours the treated stems begin to show negative curvature and by the end of 24 hours are markedly bent and slightly yellowed under the surface of application. At this time freehand sections treated with iodine show that almost all the starch in the endodermal cells beneath the surface of application has disappeared (fig. 1). The endodermal cells beneath the surface of application show radial elongation and an occasional division at the end of 30 hours. At 40 hours they have undergone active division and many show two to four derivatives. No starch can now be detected in these cells by the iodine test in freehand sections. Chloroplasts have also disappeared from cortical cells external to them. Root primordia become evident in the region of response at the end of 72-90 hours and may emerge at the end of 6-7 days. No changes are observable in the portion of the stem not covered by the mixture. The endodermal cells appear to contain

the normal amount of starch and show no signs of enlargement or of division, and the chloroplasts appear to be normal in the cortical cells outside them.

SERIES 3.—After decapitation and smearing as in series 1, pieces 2–15 mm. in length were immediately cut from the shoots and placed basal end down in petri



FIGS. 1, 2.—Fig. 1, transection from laterally treated second internode of bean 25 hours after smearing with lanolin mixture; note absence of starch in endodermal cells at left beneath surface of application and its presence at center and right where not treated. Fig. 2: *a* (6 days), apical smear with base on nutrient; both ends enlarged; *b* (6 days), basal end smeared, apical end on nutrient; base enlarged; *c* (6 days), basal end smeared and placed on nutrient; base enlarged; *d* (6 days), basal end on nutrient with 0.1 per cent aqueous indoleacetic added as thin film; basal end enlarged; *e* (6 days), apical end in nutrient with aqueous indoleacetic acid added; apical end enlarged; *f* (10 days), apical end on nutrient, basal end smeared; basal end enlarged and roots developed; *g* (7 days), apical end smeared and segment placed across gap on nutrient; both ends enlarged and base producing roots.

dishes containing a layer (about 2 mm. in thickness) of WHITE's solidified nutrient. The shorter segments were placed basal end on the medium while those 6 mm. or longer had their bases submerged. The dishes were kept on a table near an east window, where they received light of moderate intensity.

At the beginning of the experiment some of the shoots, following decapitation and smearing, were left on the plants for periods of 2, 4, 8, and 12 hours before being cut and placed on the nutrient; but since these react in the same manner as those cut off and placed in the nutrient immediately after smearing, all the later experiments were carried out as first described for this series.

The treated segments all respond in essentially the same manner. Segments 2–5 mm. long show thickening throughout their entire length, with perhaps a slightly greater amount of initial swelling at the basal end. Ultimately the apical end enlarges to a greater extent than the basal. Sections of the shorter segments show the first noticeable cellular responses in the endodermis and cortex, followed by proliferation of the cells of the cambium, phloem parenchyma, and ray cells, as has been described (1) for treated stems left on the plants. About 72 hours following treatment, root primordia begin to develop at both the apical and basal ends and may emerge at 144 hours. Usually the roots are more numerous at the apical than at the basal end, but not always, and in most instances they are limited to a narrow circular zone extending about 1–2 mm. from either end of the segment.

The longer segments, particularly those more than 8 mm., elongate noticeably during the first 24 hours after being placed on the nutrient. Elongation continues for at least 48 hours, but less rapidly during the second 24-hour period. The total increase may be as much as 25 per cent of their length at the beginning of the experiment. These longer segments show noticeable basal and apical enlargement at the end of 48 hours. The enlargement becomes more marked at 72 hours, and roots may emerge at 144 hours after treatment (fig. 2*a*). As with the shorter segments, there may be a greater amount of initial swelling at the basal end; but after several days the apical swelling becomes greater owing to the development of more callus tissue at the apex. The central region of the segment between the apical and basal ends shows little or no evident change.

Control stem segments of similar lengths treated with pure lanolin, and some with no treatment other than cutting, were placed on the nutrient and left for similar periods of time. They elongate approximately as much as the treated segments but show no other detectable gross or histological changes during periods up to 6 days.

**SERIES 4.**—This duplicates series 3, except that the segments were placed on WHITE's solidified nutrient, which was modified by omitting the sucrose. The segments remain green for 4–6 days but do not enlarge at either end nor show any detectable growth responses, other than elongation, after 96 hours.

**SERIES 5.**—This duplicates series 3 also, except that the segments were placed on WHITE's solidified nutrient, which was modified by substituting calcium sulphate for calcium nitrate and omitting the potassium nitrate. As in series 4, no swelling was observable in the segments at 6 days.

SERIES 6.—Three methods of applying the indoleacetic acid were employed. In the first, following decapitation of the plants the stems were cut off in varying lengths and the morphological bases of the segments smeared with the lanolin mixture and placed apical end down on WHITE's complete solidified nutrient. In the second the segments were smeared over their basal ends and then placed base down on the nutrient in such manner as to leave a ring of the lanolin around the base and possibly also some of the mixture on the cut surface of the base. In the third the segments were placed basal end down on the nutrient and then a thin layer of 0.1 per cent aqueous solution of indoleacetic acid was poured over the nutrient and in contact with the bases of all the segments.

The results are essentially the same for each of the treatments. The morphological base in each instance responds to the indoleacetic acid by yellowing and enlarging at about the same rate and in much the same manner as do decapitated and smeared stems when left on the plant. The morphological apex in none of these tests shows any evident changes (fig. 2*b, c, d*).

SERIES 7.—Segments of varying lengths were placed with the morphological apical end down on the nutrient and with the basal end up. A 0.1 per cent aqueous solution of indoleacetic acid was added in a thin film over the surface of the nutrient so as to come in contact with each segment. The apical ends of the segments enlarged, but the morphological base did not (fig. 2*e*). A modification of this experiment was performed by smearing the lanolin mixture over the cut surface of the apical ends of segments 5–15 mm. in length and placing the apical ends down in WHITE's solidified nutrient so as to leave a ring of the mixture around the apical end. The apical end shows enlargement in the same time intervals as when left on the plants, but no evident changes take place in the basal ends (fig. 2*f*).

SERIES 8.—A strip of the solidified nutrient was removed across the entire center of certain of the culture dishes so as to prevent the possibility of diffusion between the two portions. Segments of second internodes were then treated in two ways: (*a*) the apical ends of pieces 13–20 mm. in length were smeared with the lanolin mixture and laid across the gap so that these ends rested on the nutrient at one side of the dish and the basal ends on the other side. Both apical and basal ends enlarge, as in series 3, and at the end of 6–7 days roots develop at both ends (fig. 2*g*). (*b*) Segments of similar lengths were treated basally and also laid on the nutrient. Only the basal portions enlarge, with no indication of apical enlargement after 7 days.

### Discussion

It is evident from these experiments that the indoleacetic acid or some derivative from it moves almost entirely in a morphologically downward direction from the place of application. There is evidence from series 6, 7, and 8 that the acid or

its derivative does not move to any great extent in an acropetal direction. And for a response to occur, a certain concentration gradient of the indoleacetic acid or some derivative from it must be established downward from the surface of application. Associated with this gradient is a movement of food and nutrients upward in the internode, which enables the cells to grow, divide, differentiate, and mature. Among these substances are sugar and nitrogen needed in amounts greater than the quantities stored in the detached segments or sections.

As in the case of intact plants, when indoleacetic acid is applied to detached segments and thin sections in contact with WHITE's nutrient there is a mobilization of sugar and nitrogenous substances to the place of application. When the latter are placed in contact with a complete nutrient with no indoleacetic acid present, there appears to be little change in the stored materials in segments or sections, little further growth, and practically no tendency toward formation of tumors and roots. If indoleacetic acid is applied there is digestion of the starch present, abundant growth and proliferation, and a direct absorption of foods and nutrients from the nutrient substrate. These are mobilized to the point or surface of application and used in the development of additional cells and tissues. When treated with indoleacetic acid the cellular changes and responses are similar, whether the stems are left on the plants or detached and placed in contact with the complete nutrient.

### Summary

1. Detached segments and thin sections of the second internode of the bean, smeared apically with indoleacetic acid in lanolin and placed with the basal ends in contact with a complete nutrient, respond in essentially the same way as similarly treated stems on the plant. In addition to apical swelling and root formation, the detached segments enlarge and produce roots at the bases.
2. When treated basally and placed with the apical end on the nutrient, only the basal end enlarges. When treated apically and placed apical end on the nutrient, only the apical end enlarges.
3. Treated segments or sections placed on WHITE's nutrient lacking either sugar or nitrogen show no enlargement.
4. Segments placed with either the apical or basal end on nutrient to which 0.1 per cent of aqueous indoleacetic was added enlarge only at the end in contact with the nutrient.
5. The movement of the indoleacetic acid, or some derivative from it, takes place almost entirely in a basipetal direction with little acropetal movement.
6. When indoleacetic acid is applied to detached segments or thin sections in contact with a complete nutrient, there is digestion of the starch present, abundant growth and proliferation, and a direct absorption of foods and nutrients which are

mobilized to the region of application and used in the development of additional cells and tissues. The cellular changes and responses in the detached segments and thin sections are similar to those which take place in intact treated second internodes.

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# HISTOLOGICAL RESPONSES OF BEAN PLANTS TO TETRAHYDROFURFURYL BUTYRATE

WENDELL R. MULLISON

(WITH SEVEN FIGURES)

During the last several years a number of histological investigations have been made concerning the responses of various plants to growth-promoting substances (1, 2, 3). Tetrahydrofurfuryl butyrate when applied to bean plants as a lanolin mixture produces characteristic effects and was used in the study recorded here. The same technique has been followed as described in former studies of bean. A 2 per cent lanolin mixture of tetrahydrofurfuryl butyrate was applied terminally to the decapitated stem before the leaflets of the first compound leaf had expanded. Following the treatment, material<sup>1</sup> was collected daily for 3 weeks, the final collection being made within a month. Navashin's solution was employed as a fixative. The material was imbedded by the butyl alcohol paraffin technique and the sections cut at 12  $\mu$ .

## Investigation

### GROSS EFFECTS

Bean plants show no gross effects until about 5 days after application of the mixture. The responses are somewhat similar to those caused by indoleacetic acid, although the plants are slower to respond and the tumors are not so large. There is never more than a slight swelling of the stem immediately at or directly beneath the decapitated surface. As the tumor develops, a cavity forms in the central part of the stem; this is deepened by the upward growth of the callus. While growing upward, the callus also grows centrifugally, flaring outward over the edges of the cut surface, then beginning to grow downward, surrounding the top of the stem. At the same time centripetal growth is also taking place, resulting in complete closure at the top of the cavity, forming an irregular, flat-topped callus.

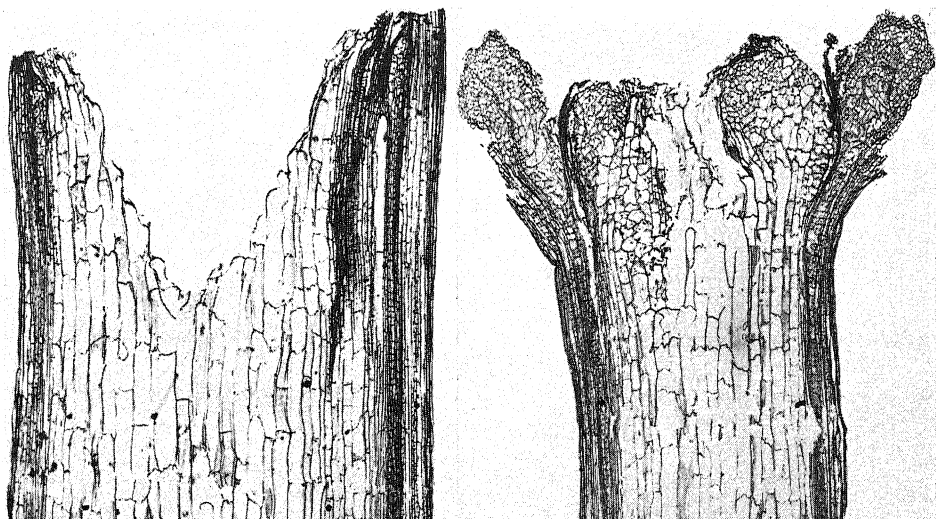
### HISTOLOGICAL EFFECTS

There is little noticeable effect until 3 days following treatment. The first sign of activity is the enlargement of some primary phloem cells, which shortly afterward become meristematic. Subsequently other tissues are involved.

There is no indication that the epidermal cells are stimulated. The paren-

<sup>1</sup> The plants used in this study were grown at the U.S. Horticultural Station, Beltsville, Maryland, during the spring of 1939 and made available for this study through Dr. E. J. Kraus.





FIGS. 1, 2.—Fig. 1 (left), 3 days after treatment: central part of pith disintegrated; initial activity in phloem. Fig. 2 (right), 7 days after treatment; great activity in pith and xylem, and some in phloem.

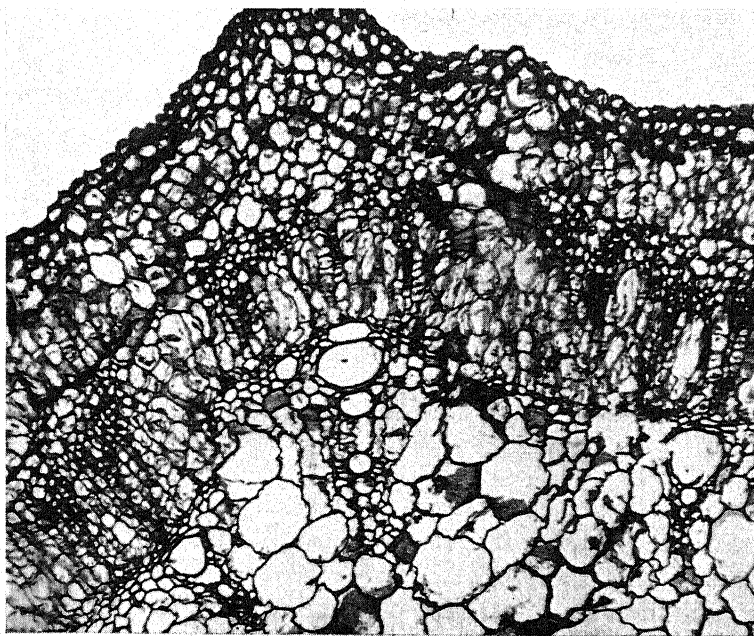


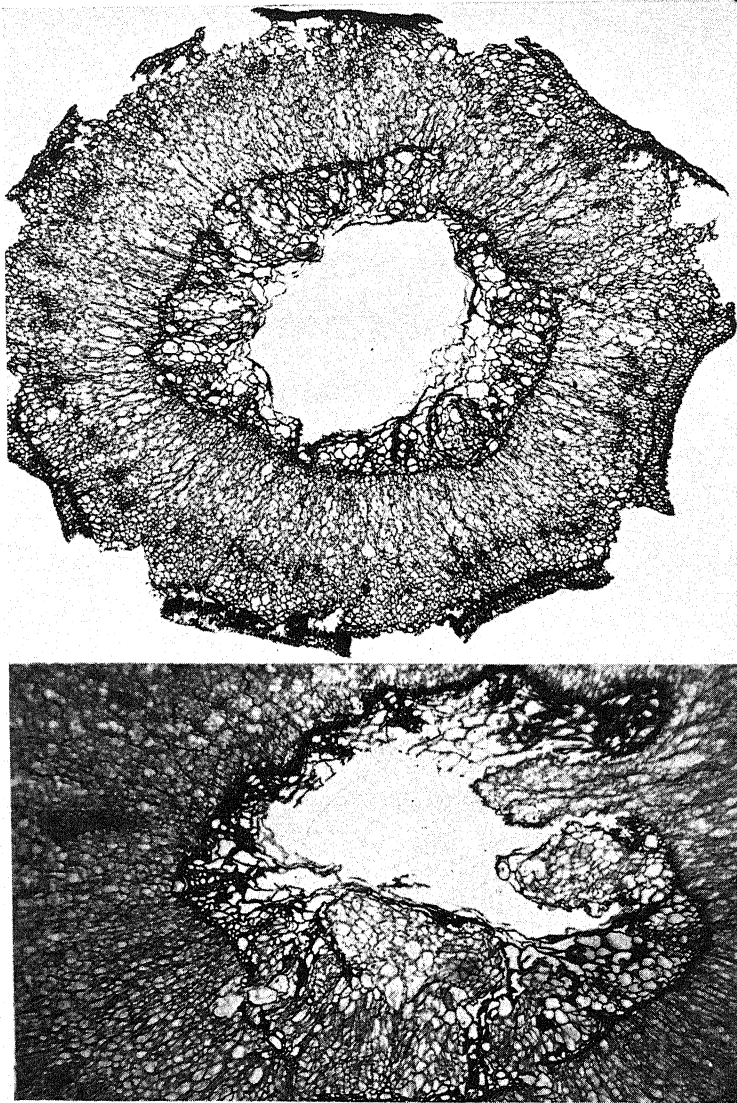
FIG. 3.—Four days after treatment: secondary xylem, phloem, and endodermis active, pericycle crushed; groups of greatly enlarged cells.

chymatous cells of the cortex also show practically no response. Later there is some sloughing of these tissues toward the tip of the stem owing to proliferation of tissues centripetal to them. The endodermis in young stages is not very active, although a few tangential divisions occur. In older stages the endodermis proliferates, giving rise to a narrow layer of parenchymatous cells. While the endodermis does not become very active, yet it responds to the stimulation at greater distances down the stem from the surface of application than do any of the other tissues. The cells of the pericycle are usually crushed or pushed from their original position by the growth of more centrally located tissues.

The phloem plays an active part in formation of the callus. Although the activity is initiated in the primary phloem, the secondary phloem and xylem become involved immediately, resulting in the formation of a wide band of meristematic tissue, most of which is derived from xylem. This band, together with meristematic areas initiated in the pith parenchyma, later forms the entire callus. The primary phloem is pushed outward, separating into small scattered units. One of the prominent features in this area in early stages is the occurrence of groups of three or four greatly enlarged cells, which are scattered at irregular intervals throughout the region. Cambial activity is slightly stimulated by the treatment, and somewhat more secondary xylem is found in the original stem portion than in untreated stems.

The xylem plays the most important role in callus formation. Delayed maturation of the metaxylem is often apparent. Sometimes cells that ordinarily would mature as metaxylem enlarge greatly and divide. At the tip of the stem the wide band of tissue derived chiefly from the secondary xylem and in small part from the phloem becomes extremely active and produces the largest part of the callus. In the callus tissue thus formed, highly disorganized vascular elements are differentiated, many of which are irregularly scattered groups of wound tracheids. Some of them, however, tie back to the vascular structure of the original stem. One characteristic feature in old callus tissue is the haphazard occurrence of groups of large cells.

Occasionally the ring of xylem about the stem is ruptured by the centripetal growth of large meristematic areas derived from the xylem parenchyma, which push aside and crush tissues in their path and penetrate into the central cavity. This cavity has appeared owing to disintegration of the pith. After the tumor has become prominent, the cavity tends to become filled with undifferentiated callus tissue, derived in part from meristematic areas arising in the parenchymatous cells of the pith adjacent to the primary xylem and in part from centripetally growing cells from the xylem as already described. The various stages in the development of the tumor are shown in the accompanying illustrations.



FIGS. 4, 5.—Fig. 4 (above), 7 days after treatment: epidermis and cortex being sloughed; scattered islands of primary phloem prominent near periphery; centripetal growth of xylem pushing through into pith cavity and meristematic areas developing in periphery of pith. Fig. 5 (below), 13 days after treatment: central cavity filling in by meristematic cells derived mainly from xylem and pith.



FIGS. 6, 7.—Fig. 6 (above), 30 days after treatment: central cavity completely filled across; vascularization of callus tissue and great amount of matured secondary xylem. Fig. 7 (below), 30 days after treatment: callus grown downward and enveloped top of stem; endodermis stimulated for considerable distance.

### Discussion

The reactions of bean plants to the several growth-promoting substances which have already been reported are sufficiently different so that in most cases they can be told apart by their gross aspects. Tetrahydrofurfuryl butyrate causes a slightly irregular, flat-topped tumor which grows downward, enveloping the stem below it for a short distance; the stem itself swells but slightly, and no adventitious roots are produced. Stems treated with naphthaleneacetic acid and alpha naphthalene acetamide also remain flat topped, but they differ greatly in amount of reaction. The former swell so much that a definite shoulder is formed about 1 cm. below the point of application, and the swelling may be apparent as far as 7 cm. below the shoulder. On the other hand, there is very little proliferation of any tissue treated with alpha naphthalene acetamide; the stem becomes very much hardened, and only a few roots are produced at the point of application.

Indoleacetic and indolebutyric acids produce large apical tumors which push up irregular warty masses overtopping a definite crown of adventitious roots; the stem becomes very swollen. The swelling of the stem caused by indolebutyric acid is the more marked of the two, extending much farther downward, and adventitious roots may be seen not only at the original point of application but 5-10 mm. below as well.

There are also marked differences in reaction of certain tissues to these five growth-promoting compounds. In all cases there is little or no proliferation in the epidermis and pericycle, although the cell walls of the latter are thickened by alpha naphthalene acetamide.

Reactions of the cortical cells range from no activity through marked proliferation. Tetrahydrofurfuryl butyrate causes no reaction; indoleacetic acid causes simple cell enlargement; indolebutyric acid causes marked enlargement; alpha naphthalene acetamide causes thickening of the cell walls; and naphthaleneacetic acid causes great activity.

After applications of tetrahydrofurfuryl butyrate there is moderate activity of the endodermis, with no formation of vascular tissues or root primordia. With all other compounds the endodermis shows great activity, vascular bundles becoming differentiated in its derivatives. In the case of indoleacetic acid the endodermal derivatives give rise to some of the root primordia, while in the case of indolebutyric and naphthaleneacetic acid it is involved along with other tissues in root formation.

When treated with tetrahydrofurfuryl butyrate the phloem becomes a fairly active tissue, producing part of the callus and containing some highly disorganized groups of tracheids. Alpha naphthalene acetamide causes but little activity in the phloem. The other three compounds cause great activity there, producing a mass of tissue with many vascular strands running through it.

Tetrahydrofurfuryl butyrate slightly stimulates cambial development and causes it to produce a little more secondary xylem than in the untreated stem. The other four cause pronounced proliferation. In these latter cases differentiation of cambial derivatives is delayed, except in the case of alpha naphthalene acetamide, where the production of considerable secondary xylem is brought about

TABLE 1  
HISTOLOGICAL EFFECTS OF GROWTH-PROMOTING COMPOUNDS ON BEAN

TISSUE	RESPONSE		
	TETRAHYDROFURFURYL BUTYRATE	INDOLEACETIC ACID*	ALPHA NAPHTHALENE ACETAMIDE
Epidermis.....	No activity	Little activity	No activity
Cortex.....	No activity	Disappearance of chloroplasts and starch grains; some cells enlarged	Thickening of cell walls
Endodermis.....	Slight activity but no differentiation of vascular bundles	Great activity; differentiation of vascular bundles	Moderate activity; differentiation of vascular bundles
Pericycle.....	Little activity	Little activity	Fibers much thickened
Phloem.....	Activity; produces part of callus	Great activity; differentiation of vascular bundles	Little activity
Cambium.....	Stimulated; produces more secondary xylem than in untreated plants but not nearly so much as alpha naphthalene acetamide	Divides rapidly but differentiation of derivatives delayed	Most active tissue; much secondary xylem
Xylem.....	Most active tissue; produces most of callus	Little activity	Little activity; increased thickness of cell walls
Pith rays.....	Very active but no differentiation of vascular bundles	Very active; differentiation of vascular bundles	Activity
Pith.....	Cells adjacent to primary xylem active	Eventually very active and produces most of callus	Slower than indoleacetic and not so active; differentiates a few vascular bundles
Tissues involved in root formation..	None	{Pith rays and phloem† Endodermis	Pith rays

\* Indolebutyric and naphthaleneacetic acids produce about the same response as indoleacetic acid.

† Most of the roots are derived from these tissues.

by the cambium, the most active tissue. Tetrahydrofurfuryl butyrate causes the cambial derivatives that would mature as xylem to become meristematic and to play an extensive role in callus formation, while in the other three the xylem plays only a slight role.

In all cases the pith rays become highly meristematic. Indoleacetic and indolebutyric acids cause differentiation of many vascular bundles in the pith rays.

The others produce none. Tetrahydrofurfuryl butyrate kills the cells of the central portion of the pith, but later the parenchyma of the pith adjacent to the primary xylem becomes very active and in conjunction with the xylem fills up the cavity with parenchymatous cells. Naphthaleneacetic acid causes little activity in the pith at any time. In the other three cases the pith is slow to respond, but eventually becomes quite active and produces a mass of callus in which vascular bundles become differentiated.

Roots are never produced at the tumor or near it after application of tetrahydrofurfuryl butyrate. In the other four cases root primordia are produced. Alpha naphthalene acetamide initiates root production solely in the pith rays. Naphthaleneacetic acid also stimulates the pith rays to produce root primordia, and in addition it causes their occasional formation in the endodermis. Indoleacetic acid causes root production mostly in the pith rays and phloem, and also to some extent in the endodermis, while indolebutyric acid produces roots in which the endodermis and phloem are both involved; it may also form roots in the pith rays.

The similarities and differences in the histological behavior pattern of bean to the growth-promoting compounds thus far investigated are summarized in table 1.

### Summary

There are striking differences between the reaction of bean to tetrahydrofurfuryl butyrate and the other substances thus far investigated. The most prominent is the complete absence of root primordia arising either as a part of the tumor or near it. The xylem is the most active tissue, which with its derivatives forms by far the largest part of the callus, although the phloem is also involved. The callus, while having differentiated a considerable number of wound tracheids, some of which tie back with the vascular tissue of the stem, is largely composed of parenchymatous cells, scattered among which are groups of extremely large cells and areas of meristematic activity. The cavity formed by disintegration of the central region of the pith tends to be filled in with parenchymatous cells derived in part from the pith and in part from the xylem. Cambial activity is stimulated and produces a little more secondary xylem than in untreated stems. There is a moderate amount of activity in the endodermis, with no differentiation of vascular tissue. The endodermis, however, responds to the stimulation of tetrahydrofurfuryl butyrate farther down the stem from the surfaces of application than does any other tissue. Another striking difference is the localization of the response within 1-3 mm. of the site of application.

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# NITROGEN CONTENT OF SOUND AND DECAYED CONIFEROUS WOODS AND ITS RELATION TO LOSS IN WEIGHT DURING DECAY

R. E. HUNGATE

Nitrogen changes during wood decay are of interest not only as they affect forest soil fertility but also from the standpoint of the physiology of the fungi concerned. The amount of nitrogen in wood is extremely small. In the present studies it has been found in *Pinus monticola* to average 0.048 per cent of the dry weight of the sapwood and 0.031 per cent of the heartwood. In spite of such low contents, many investigations (2, 7, 12) show that pine wood may lose more than one-third of its weight during attack by pure cultures of wood-destroying fungi. If the nitrogen content of the woods used was about the same as that found in the woods of the present report (FINDLAY (2) gives a value of 0.04 per cent), it is evident that the quantity of carbon compounds decomposed is almost 700 times the amount of nitrogen utilized, unless unknown additional nitrogen sources were available to the fungi. This is very much larger than the ratio of 30 to 1 reported for other cellulose-destroying fungi (3, 6). The results of FINDLAY (2) and SCHMITZ and KAUFERT (7) indicate that addition of a suitable nitrogen source to the wood increases the rate of decay in laboratory cultures. It has seemed of interest to investigate the natural destruction of wood by fungi and to determine whether the nitrogen in the wood is the sole source of this necessary element.

## Material and methods

Samples of sound and decayed wood of several species of coniferous trees were collected and used for determinations of dry weight, specific gravity, and nitrogen content. Data on specific gravity were obtained in order to allow for any increase in percentage of nitrogen due to a decrease in other materials. Since the specific gravity is known, it is possible to compare the nitrogen content per volume of sound wood with an equal volume of decayed wood.

There are changes in the volume of wood during decay (5), particularly during the later stages, so that comparison on the basis of equal volumes may introduce an error. In obtaining samples of decayed wood for the present analyses, only those which seemed to have retained their original shape and were without large cracks and fissures were selected. This not only reduced errors due to volume changes but also assured that the samples remained intact during the determinations of specific gravity. Although no data on volume change during decay were

obtained, any change would probably be in the direction of decreased volume and therefore would give higher values for the percentage of nitrogen in a unit volume of decayed wood than for the same volume of sound wood, provided there was no change in absolute amount.

The woods were from virgin, over-mature forests on the slopes adjacent to the east shore of Priest Lake, Idaho, in the vicinity of Lion Head Bay. Samples of sound and rotten western white pine (*Pinus monticola*), western hemlock (*Tsuga heterophylla*), western red cedar (*Thuja plicata*), and white fir (*Abies grandis*) were obtained. An ax or saw was used to cut out a portion of the selected log to give a sample about 6–8 inches long (with the grain) and containing both sapwood and heartwood. Care was taken to avoid inclusion of insects or their borings in the sample. When possible, the wood was cut from the rotten logs at a distance of 6–10 feet from the point of contact with the ground. This precaution decreased the possibility of nitrogen absorption from the soil by such physical methods as capillarity. It was also desired to investigate natural wood decomposition under conditions where additional nitrogen was not readily available, in order to determine the magnitude of weight loss under these circumstances. If soil nitrogen was used for the decay of the wood thus selected, it must have been transported for a considerable distance.

Samples were immediately dried in the warming pan of a kitchen stove and after shipment to Austin, Texas, were dried overnight at a temperature of 106° C. They were allowed to remain in air for some time, were weighed, and their specific gravity determined by weighing the amount of ethyl alcohol (s.g. 0.80) which they displaced when immersed in a bucket full of alcohol, provided with an overflow spout. Alcohol was selected because of its low surface tension and because it speedily penetrated the wood. Before immersing the sample in the test bucket, it was placed in another bucket of alcohol and allowed to stand until it was saturated. This prevented a false value due to uptake of liquid by the unwetted wood. Transfer from the preliminary to the test alcohol was accomplished quickly so that no alcohol was lost from the interior of the sample. The displaced alcohol was collected and weighed quickly so that the error due to evaporation could be neglected. A determination on a sample using alcohol was checked with mercury and similar results obtained. The alcohol was analyzed for its nitrogen content before and after use and it was found to be negligible in both cases. Thus no error was introduced owing to nitrogen added or removed by the alcohol.

After the specific gravity determination, the sample was again dried at 106° C. and weighed. There was no significant change from the first weight. It was ground in a Wiley mill until it would pass a 1-mm. sieve. The ground wood was collected in paper bags and set aside for the determinations. Thus the nitrogen analyses were made after the wood had come to more or less of an equilibrium with the air.

Humidity changes would cause some variations in the weight of the sawdust (13), but this error was estimated to be less than the error of 4 per cent in determining nitrogen.

The low percentage of nitrogen in wood makes it difficult to determine the total nitrogen accurately, since a large sample must be used in order to get sufficient nitrogen to measure. The use of micro-methods does not solve the problem, since these methods are adapted to minute amounts of materials containing fairly large percentages of nitrogen but not to material containing minute percentages.

The Dumas, Kjeldahl, and ter Meulen methods for total nitrogen have been considered during the course of the present work. The Kjeldahl has given the most reproducible results. In the earlier analyses the ter Meulen methods were used (8, 9, 10) and occasionally gave (with duplicate samples) values which differed by as much as 25 per cent. The several modifications of the ter Meulen technique were tried but none was particularly satisfactory. The disagreement between duplicate samples was considerably greater than the error in titration.

Dr. A. J. HAAGEN-SMIT at the California Institute of Technology kindly investigated the feasibility of determining nitrogen by the Dumas method when it is present in such small percentages and found that the errors arising from the use of the necessarily large sample are so great as to make the method unsuitable. In view of the superiority of the Dumas method for total nitrogen, it is unfortunate that it has not been possible to check the Kjeldahl results against it. The values obtained by the ter Meulen and the Kjeldahl methods are of the same magnitude, however, and it is probable that the found nitrogen values are not far from the true values.

The usual macro-Kjeldahl analysis was found to involve errors owing to the large amount of fluid in which the final titration was accomplished. It was also time-consuming. After a few experiments it was decided that best results could be obtained by digesting samples of semi-micro size (200 mg.) and distilling and titrating on a micro-scale. The 200-mg. sample was placed in a 100-cc. Kjeldahl digestion flask with 1 gm. of potassium sulphate, 0.1 gm. mercury, and 3 cc. of concentrated sulphuric acid. Digestion was completed in 20-30 minutes; and after cooling, the digest was diluted with 10 cc. of cold water.

A Pregl micro-still was constructed, similar to the usual apparatus, except that a bent tube to serve as an additional trap for sodium hydroxide droplets was inserted in the main flask, so that the vapors passed through it before entering the customary trap. This arrangement was found to be so effective that rapid steam distillation for 5 minutes through an ammonia-free strongly alkaline solution resulted in driving over basic materials equivalent to less than 0.02 cc. of 0.006 normal acid. The distillation was carried out as usual after addition of 10 cc. of the NaOH-Na<sub>2</sub>S mixture, except that the ammonia driven off was titrated as it

TABLE 1  
NITROGEN CONTENT AND SPECIFIC GRAVITY OF WOOD SAMPLES

WOOD AND SAMPLE NO.		SOUND				SAMPLE NO.	ROTTEN			
		SAPWOOD		HEARTWOOD			SAPWOOD		HEARTWOOD	
		Mg. N. CC.	SPEC. GRAV.	Mg. N. CC.	SPEC. GRAV.		Mg. N. CC.	SPEC. GRAV.	Mg. N. CC.	SPEC. GRAV.
Pine	1...	0.17	0.419	0.12	0.434	9	0.22	0.329	0.17	0.443
	3...	0.23	0.497	0.18	0.506	10	0.19	0.426	0.14	0.425
	18...	0.32	0.501	0.22	0.548	13	.....	.....	0.18	0.394
	37...	0.21	0.441	0.14	0.526	14	0.24	0.343	0.15	0.387
	38...	0.30	0.536	0.21	0.551	19	0.17	0.303	0.11	0.420
	39...	0.22	0.497	0.15	0.561	22	0.17	0.344	0.13	0.442
	40...	0.26	0.541	0.14	0.543	30	0.20	0.298	0.15	0.488
	42...	0.19	0.465	0.11	0.460	32	0.14	0.219	0.10	0.357
	48...	0.18	0.380	0.15	0.493	33	0.16	0.346	0.12	0.370
	51...	0.18	0.426	0.14	0.428	44	0.29	0.480	0.20	0.412
	.....	.....	.....	.....	49	0.26	0.375	0.17	0.355	
	.....	.....	.....	.....	50	0.18	0.424	0.16	0.486	
Hem- lock	2...	0.24	0.495	0.17	0.504	12	0.19	0.328	0.19	0.436
	11...	0.23	0.492	0.17	0.494	20	0.17	0.413	0.14	0.397
	16...	0.27	0.485	0.20	0.505	21	0.16	0.292	0.15	0.288
	17...	0.22	0.424	0.23	0.471	25	0.17	0.385	0.18	0.388
	24...	0.19	0.564	0.17	0.564	26	0.23	0.382	0.16	0.428
	27...	0.21	0.486	0.18	0.479	28	0.23	0.332	0.18	0.341
	29...	0.20	0.500	0.16	0.521	34	0.25	0.392	0.20	0.407
	35...	0.21	0.530	0.19	0.481	41	0.15	0.331	0.24	0.329
	.....	.....	.....	.....	43	0.27	0.439	0.22	0.423	
	.....	.....	.....	.....	45	0.26	0.376	0.18	0.404	
	.....	.....	.....	.....	46	0.29	0.344	0.22	0.406	
	.....	.....	.....	.....	47	0.32	0.452	0.16	0.471	
Cedar	5...	0.18	0.370	0.18	0.401	7	0.33	0.300	0.20	0.424(s)*
	6...	0.18	0.381	0.19	0.384	8	0.15	0.275	0.14	0.319(s)
	15...	0.20	0.316	0.19	0.410	52	0.37	0.378	0.21	0.419(ps)
	23...	0.24	0.382	0.16	0.402	55	0.25	0.264	0.19	0.334(ps)
	57...	0.24	0.290	0.19	0.324	56	0.27	0.305	0.17	0.357(ps)
	62...	0.19	0.394	0.17	0.444	61	0.16	0.331	0.16	0.338(ps)
	65...	0.22	0.312	0.16	0.388	63	0.20	0.346	0.15	0.289(ps)
	68...	0.16	0.328	0.12	0.364	64	0.16	0.301	0.15	0.305
	70...	0.24	0.392	0.13	0.395	69	0.22	0.354	0.13	0.353(ps)
	71...	0.37	0.433	0.23	0.470	.....	.....	.....	.....	.....
	74...	0.30	0.375	0.18	0.433	.....	.....	.....	.....	.....
Fir	60...	0.43	0.411	0.14	0.377	66	0.17	0.204	0.12	0.378
	73...	0.16	0.492	0.13	0.393	72	0.26	0.364	0.24	0.402
	77...	0.38	0.497	0.17	0.511	75	0.17	0.296	0.26	0.280
	79...	0.17	0.354	0.14	0.347	76	0.43	0.306	0.31	0.479
	80...	0.34	0.504	0.18	0.519	78	0.24	0.242	0.18	0.260

\* (s) sound; (ps) partially sound.

came over instead of being trapped in an excess of acid and back titrated. With this modification it was possible to keep the volume of liquid in the titration vessel down to less than 10 cc., and the accuracy of the titration was accordingly increased. This particular change was taken over from the ter Meulen method, and it not only increased the accuracy but also was quicker; the point at which no more ammonia came over could be accurately detected and the distillation stopped at that point, instead of continued over for an additional margin of safety. Initially some difficulty may be experienced in catching the first rush of ammonia without letting the titration vessel become too alkaline, but as soon as the approximate amount of nitrogen in the sample is known it is easy to maintain a suitable excess during the critical period.

Nitrogen determinations were always run in duplicate, and with few exceptions the duplicates agreed to within 4 per cent of the total nitrogen when the latter was 0.05 per cent. This absolute error of 4 gammas of nitrogen is about equal to one drop of the acid used. The agreement was achieved not only by the writer but after a few trials also by students with little previous training. With each set of nitrogen determinations a blank was run on the reagents and a correction made. Further refinements probably would have increased the accuracy, but it was not thought necessary for the present studies, using averages of many samples.

### Analytical results

Table 1 gives the results of the determinations. Each nitrogen value is the average of duplicate determinations.

The mean nitrogen content and the probable error of the mean for each species in table 1 have been calculated and are assembled in table 2.

The results obtained with pine, hemlock, and cedar show no significant change in the total nitrogen content during decay. The rotten sapwood of the fir shows less nitrogen than the sound sapwood, but the error here is larger owing to the smaller number of samples and the greater variation. The figures for fir heartwood show an increase in nitrogen content with decay. The difference is not large, however, and coupled with the fact that the rotten sapwood shows a decrease in nitrogen, the evidence for a total increase is not convincing.

In general the analytical results indicate that there is no significant change in nitrogen during decay. This agreement between the nitrogen values for sound and decayed wood also suggests that the decayed samples had not undergone any significant shrinkage in volume. It is true that a similarity in the nitrogen content between sound and decayed wood might result if the loss in nitrogen during decay exactly balanced any gain. Unless the loss and gain were linked in some way, however, it does not seem probable that they would be exactly equal. The close agreement between the nitrogen content of sound and rotten wood in these analyses is

better interpreted as nitrogen retention. If this is correct, the nitrogen used by the fungi attacking the wood cannot be greater than the total nitrogen content.

From the mean values for the specific gravity of the sound and decayed samples, the amount of materials (principally non-nitrogenous) disappearing during decay can be calculated. The average specific gravities are given in table 3. The difference column gives the average grams of wood per cubic centimeter that dis-

TABLE 2  
SUMMARY OF DATA ON NITROGEN CONTENT  
AVERAGE NITROGEN IN MG./CC.

WOOD	SAPWOOD		HEARTWOOD	
	SOUND	ROTTEN	SOUND	ROTTEN
Pine.....	0.226±0.010	0.202±0.009	0.156±0.007	0.148±0.006
Hemlock.....	0.221±0.006	0.224±0.010	0.184±0.005	0.185±0.006
Cedar.....	0.229±0.012	0.234±0.017	0.173±0.006	0.167±0.006
Fir.....	0.296±0.033	0.254±0.027	0.152±0.006	0.222±0.020

TABLE 3  
AVERAGE SPECIFIC GRAVITIES OF WOOD SAMPLES IN GM./CC.

WOOD	SAPWOOD			HEARTWOOD		
	SOUND	ROTTEN	DIFFERENCE	SOUND	ROTTEN	DIFFERENCE
Pine.....	0.470	0.353	0.117	0.505	0.415	0.090
Hemlock.....	0.497	0.372	0.125	0.503	0.393	0.110
Cedar.....	0.361	0.317	0.044	0.401	0.349	0.052
Fir.....	0.452	0.282	0.170	0.429	0.360	0.069

appeared during decay. This can be compared with the nitrogen per cubic centimeter of sound wood, as has been done in table 4.

The values of the ratio for pine, hemlock, and fir are rather similar, whereas for cedar lower values are found. These low values can be ascribed in part to certain difficulties in securing samples of the wood of this species. The central heartwood is not resistant to decay and in standing and fallen logs is usually rather rotten. The fungus attack in this central portion results in such checking and shrinking that comparisons on the basis of volume are impossible, thus preventing use of these well-decayed portions. The outer heartwood is extremely resistant, and in some cases logs which have been down for as long as 100 years show little sign of extensive decay (5). When decay does appear it is usually not uniform but is con-

fined to narrow strips between areas of sound wood. In collecting the samples no attempt was made to separate the rotten from the sound portions, since it was thought that the difference in fungus attack might be due to differences in the nitrogen in local areas of the wood, and thus a selection might give high or low nitrogen values which should not be compared with the average nitrogen values determined for sound wood. Table 3 shows that the weight of cedar heartwood decomposed is considerably less than for the other species. Since only part of the wood had decomposed, and yet the nitrogen in the sound part has been included in the carbohydrate/nitrogen ratio, the value of the ratio would be expected to be low. The opposite difficulty was experienced with cedar sapwood, in that it decays very quickly and some of the samples of down logs classified as sound and contain-

TABLE 4  
RATIO BETWEEN COMPOUNDS DISSIMILATED AND TOTAL NITROGEN

WOOD	SAPWOOD			HEARTWOOD		
	WOOD DISAPPEARING (MG.)	N/CC. (MG.)	RATIO	WOOD DISAPPEARING (MG.)	N/CC. (MG.)	RATIO
Pine.....	117	0.226	518	90	0.156	576
Hemlock.....	125	0.221	565	110	0.184	597
Cedar.....	44	0.220	192	52	0.173	300
Fir.....	170	0.206	575	69	0.152	454

ing sound heartwood had the sapwood decayed. Thus the calculated weight of wood disappearing during decay is lower than the real loss and the carbohydrate/nitrogen ratio is correspondingly reduced.

The ratios for fir are less reliable than those for pine and hemlock because of the fewer samples analyzed and their greater variation. The increase in nitrogen during decay in the heartwood and the decrease in the sapwood suggest the possibility that nitrogen was transferred from sapwood to heartwood. A conduction of nitrogen for a distance of about 9 inches from soil through wood has been detected in a laboratory culture of termites on *Pinus radiata* in which the wood was attacked by fungi. It is conceivable that a similar transfer might have occurred across the fir logs from the sapwood to the heartwood. If so, then the amount of nitrogen used during the decay of either sapwood or heartwood cannot be accurately ascertained with the available data, and the carbohydrate/nitrogen ratio is correspondingly less reliable. The values for fir, however, are of the same general magnitude as for pine and hemlock.

### Discussion

One of the numerous factors affecting the relative nitrogen content of sound and decayed wood is the amount of nitrogen transferred into the fruiting bodies of the fungi. The sporophores contain a larger percentage of nitrogen than is found in the wood, and if there are no additional sources the nitrogen content of the wood might be expected to be low when fruiting bodies have been formed. If, however, the nitrogen content of the food of fungi is low, the nitrogen content of the fungus body is reduced (11). FINDLAY (2) gives a nitrogen content of 0.68 per cent for the mycelium of *Polystictus versicolor* growing on wood. If the nitrogen in the fruiting bodies on wood were about this same percentage, it would take 14 gm. of wood with a nitrogen content of 0.05 per cent to contain sufficient nitrogen for 1 gm. of fungus. With an error of 4 per cent in determining nitrogen, a draining of nitrogen from the wood by 1 gm. of fungus would not be detected if the area for nitrogen collection included more than 350 gm. In all the logs selected for the present analyses the weight of the wood was estimated to be much more than 350 times the weight of the sporophores. Thus any accumulation of nitrogen in the fruiting bodies would not be detected in these analyses. Two samples of rotten hemlock showed conspicuous accumulations of mycelium at many spots. One of these, no. 46, contained a higher than average amount of nitrogen per cubic centimeter, 0.29 per cent, but the other, no. 20, was lower than average, 0.17 per cent.

The fact that prominent fruiting bodies are often formed demonstrates a capacity on the part of the fungi to transport nitrogenous materials for some distance through the wood. Since decaying logs are often partially imbedded in the ground it is possible that soil nitrogen may also be transported. Common observation indicates that the portion of the log immediately in contact with the ground is usually the most decayed. It is probable that nitrogen as well as moisture conditions is concerned, and as just stated, this has been observed in a laboratory culture. The similarity between the nitrogen content of sound and decayed logs in the present analyses indicates that soil nitrogen was not transferred as far as the points from which samples were taken, at least not in detectable amounts. Since precautions were taken to obtain rotten samples at a considerable distance from ground contacts, a transfer of nitrogen into wood in close proximity to the soil would not have been detected.

The experiments of FINDLAY (2) and of SCHMITZ and KAUFERT (7) bear directly on the importance for decay of the nitrogen in wood. FINDLAY compared the loss in weight of Sitka spruce with and without added nitrogen when attacked by *Trametes serialis*. Addition of 0.5 per cent ammonium nitrate gave a loss of 15.08 per cent, whereas 10.62 per cent was dissimilated in the control without added nitrogen. One per cent peptone increased the loss from 25.83 to 40.82 per cent.



The amounts of added nitrogen based on the weight of wood were 0.175 per cent for the ammonium nitrate and 0.167 per cent for the peptone. The nitrogen content of the wood was 0.04 per cent. SCHMITZ and KAUFERT found similar effects. Addition of asparagine, in which the nitrogen amounted to about 0.04 per cent of the weight of the Norway pine sapwood, increased the weight loss to 35.5 per cent when the controls without added nitrogen showed a loss of 33 per cent. With 0.2 per cent added nitrogen as asparagine the loss was 46.7 per cent; with 0.4 per cent the loss was 47.5 per cent. Ammonium nitrate in which the nitrogen amounted to 0.066 per cent of the weight of the wood increased the loss to 37.8 per cent, but higher concentration decreased the rate of decay. The naturally occurring nitrogen in the wood was not determined but probably was about 0.04-0.05 per cent.

The experiments with added nitrogen suggest that growth of the fungi of wood decay is limited by the amount of available nitrogen in the wood. They also emphasize the extreme economy with which the nitrogen in the wood is utilized. Additional nitrogen equal in amount to that already present increased the weight loss only one-tenth, and addition of more than four times the amount increased it only one-half. From these figures it can hardly be concluded that the fungi are in a critical state of nitrogen starvation.

Higher values for the carbohydrate/nitrogen ratio might have been observed in the present analyses had it been possible to work with samples that were more completely decayed. The data on specific gravity for pine and hemlock indicate that about 25 per cent of the wood had disappeared. The data of others indicate that as much as 33 per cent may disappear in a pure culture. If it were possible to get accurate specific gravity determinations on samples which were more completely decayed, the values of the ratio of carbohydrate to nitrogen would probably be larger than those calculated from the present analyses.

Another factor which might have influenced the value of the carbohydrate/nitrogen ratio was the method of selection of the sound wood samples. Since it was preferred not to disfigure the living trees, most of the samples were from down logs. Standing or recently fallen dead trees were selected; and if after cutting into the log the wood appeared to have the color, texture, and strength characteristic of sound wood, it was accepted as suitable. Since the initial stages of fungus decay are not always macroscopically evident (4), it is possible that some of the sound samples had undergone a slight loss of weight due to fungus attack. This would make the carbohydrate/nitrogen ratio somewhat lower than if completely undecayed wood were used. Where samples of both living and fallen dead trees were collected there was no indication that the fallen ones had a significantly lower specific gravity, except in the case of the cedar sapwood already mentioned. Since it is improbable that the initial mycelium of infection would be more effective in bringing nitrogen into the wood than the later mycelium, these questions as to the

soundness of the sound samples do not affect the conclusion that the amount of nitrogen in the wood is unchanged during decay.

It should be emphasized that the calculated carbohydrate/nitrogen ratios are not the ratio between the carbohydrate utilized and the nitrogen utilized, but are rather the ratio between the carbohydrate disappearing as loss in weight and the total nitrogen present. If, as the work of BRAY and ANDREWS (1) indicates, some of the carbon compounds in the wood are attacked and changed by the fungi without necessarily being decomposed to carbon dioxide and water, then the ratio between carbon compounds used by the fungi to total nitrogen in the wood will be larger. Similarly, if some of the nitrogen in the wood is not used by the fungi, then the ratio between carbon compounds used and nitrogen used would be still larger. All these considerations point to the fact that the ratios calculated are conservative estimates of the economy with which wood-destroying fungi are able to use nitrogen in the destruction of the carbohydrate materials.

The high carbohydrate/nitrogen ratio might be interpreted as indicating a cycle of nitrogen in the wood, in which this material is used over and over by various organisms. Apparently, however, a cycle in which numerous organisms are concerned is not necessarily a factor in the observed nitrogen efficiency, since pure cultures of fungi are able to decompose as much wood as was calculated from these analyses to have disappeared during natural decay. If there is repeated use of nitrogen, the repetition is accomplished by the same organism. It is interesting to speculate on whether such a physiological mechanism may explain the nitrogen economy observed.

Although apparently unable to fix atmospheric nitrogen or to transport this material over very great distances from soil into wood, although living in a medium containing occasionally as little as 0.25 mg. of nitrogen per gram of other materials, yet the wood-destroying fungi are able to penetrate and spread, dissimilating relatively huge quantities of the non-nitrogenous materials, and ultimately forming prominent fruiting bodies. The problems presented in the decomposition of a resistant carbohydrate material poor in nitrogen have apparently been met during the course of evolution of this group of fungi, and this may account in part for their unique position as the prime agents of wood decay.

### Summary

1. A satisfactory method for the determination of total nitrogen in wood is described.
2. Determinations of specific gravity and nitrogen content for sound and decayed woods indicate that there is little change in the total nitrogen of wood during decay.

3. Comparisons between the amount of wood disappearing during decay and the maximum amount of nitrogen available indicate that the amount of wood is 500-700 times as great as the total nitrogen available.
4. Considerations of the effects of added nitrogen indicate that the nitrogen present is utilized more effectively during decay than is that added.
5. The efficiency of certain fungi in utilizing nitrogen may explain in part their great success in destroying wood.

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# STARCH HYDROLYSIS IN BEAN LEAVES AS AFFECTED BY APPLICATION OF GROWTH-REGULATING SUBSTANCES

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(WITH ONE FIGURE)

## Introduction

In a previous investigation (7) it was noted that the rate of starch hydrolysis in attached bean leaves kept in darkness was increased as a result of application of naphthaleneacetic acid. It was also observed that the sugar content of leaves kept in darkness increased appreciably for several hours following treatment, while that of control leaves steadily decreased. When leaves were first depleted of carbohydrates, then treated and illuminated by means of natural light, they accumulated carbohydrates more slowly than did untreated leaves under comparable conditions.

In view of the fact that naphthaleneacetic acid affected the rate of starch hydrolysis and the sugar content of leaves, it was decided to study these responses in more detail and to determine the relative effectiveness of several other growth-regulating substances in inducing similar responses. Seven compounds were selected as representative of those that induce various histological and physiological responses in plants (1, 2, 3, 4, 6, 8, 9), including: phenylacetic, beta indole-3-acetic, gamma (indole 3)-n-butyric, beta indole 3-propionic, alpha naphthaleneacetic, and beta naphthoxyacetic acids, and alpha naphthalene acetamide. Emulsions containing these substances were sprayed on attached bean leaves, and their relative effectiveness in increasing the rate of starch digestion above that of comparable control leaves, during a period of darkness, was determined by quantitative analyses. The effect of these substances on the rate of starch digestion in relatively young as compared with mature leaves was studied, and the effect of indoleacetic acid on the sugar content of relatively young leaves kept in darkness was determined.

## Methods

Attached heart-shaped leaves of kidney bean plants, Calapproved variety, were used. A great number of plants were grown under greenhouse conditions in soil contained in 4-inch clay pots for a period of 2-4 weeks. Plants were carefully selected from this supply for the experiments. Relatively bright and clear weather prevailed during the experiments, so that it was not difficult to obtain leaves with an appreciable amount of reserve carbohydrate in the form of starch. In order to

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accelerate the storage of starch in the heart-shaped leaves, all portions of the plants above the second node were removed, as was done in previous experiments (7). In treating the leaves, lanolin-water emulsions containing the different growth-regulating substances were sprayed on the upper surfaces (7).

Composite samples of fifty leaves which had first been dried in a well-ventilated oven at 80° C., washed five times with ether, ground to 100 mesh, and finally re-dried in a vacuum oven, were used for chemical analyses.

Sugar in the dried samples was determined in duplicate as previously described (7). The combined starch and dextrin was determined by first removing sugars from a weighed amount of the powder by means of repeated extractions with 80

per cent alcohol. The starch was then gelatinized by holding the temperature of the wet sample at 75°–80° C. for 30 minutes. After cooling, 1 ml. of fresh, filtered saliva, which had been diluted with an equal volume of water, was added, together with 10 drops of toluene, and the starch digested for 30 minutes at 35°–38° C. The sample was again heated to 75°–80° C. for 30 minutes, cooled, fresh saliva added and again digested for 30 minutes. Periods of alternate heating and digesting were continued until all the starch was

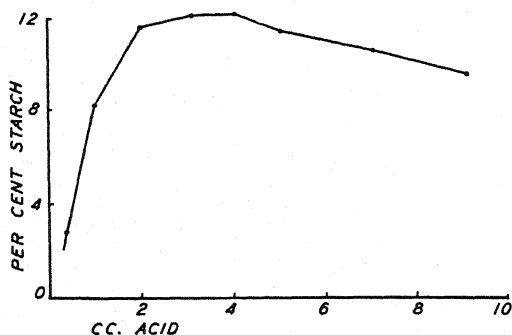


FIG. 1.—Effect of heating aliquots of a solution containing dextrin and products of saliva digestion for 20 minutes at 112°–114° F. with different amounts of hydrochloric acid. Reducing power of solutions after hydrolysis expressed in terms of combined percentages of starch and dextrin in leaf sample.

hydrolyzed, as shown by the iodine tests. The sample was then filtered and cleared with lead acetate. A 50-ml. aliquot of the cleared solution was placed in a 100-ml. volumetric flask, together with 3 ml. of concentrated hydrochloric acid. The acidified solution of dextrin and the products of saliva digestion were then autoclaved for a period of 20 minutes at a temperature of 112°–114° C.

The reducing power of the solution, following hydrolysis of dextrin and the products of saliva digestion, depended largely upon the conditions under which the hydrolysis was carried out. Although the solution was heated for only 20 minutes, relatively low figures were obtained when 10 ml. of acid was used in 50 ml. of the solution to be hydrolyzed, apparently because some of the sugar was caramelized. In order to determine the optimum conditions for hydrolysis, various amounts of acid were used, and the solutions were heated for 20 minutes at a temperature of 112°–114° C. The results of this experiment show that maximum reducing power resulted when the solution containing dextrin and the products of

saliva digestion was hydrolyzed by adding 3 ml. of acid to 50 ml. of solution (fig. 1). The pH of the cleared solution with this amount of acid added was approximately 0.35.

In order to determine whether 20 minutes was sufficient time for heating, quadruplicate samples were prepared and 3 ml. of acid was added to each. Two were then heated for a 20-minute period, while the others were heated for 1 hour. No difference was noted in the reducing power of the final solutions.

These data show that maximum reducing power resulted when 3 ml. of acid was added and the solutions heated at  $112^{\circ}$ – $114^{\circ}$  C. for 20 minutes. These conditions were considered optimum for this method of hydrolysis, and were therefore used in all the starch determinations. The reducing power of the sugar solutions following acid hydrolysis was determined as previously described (5). As a matter of simplicity the combined starch and dextrin content of samples is reported in the tables as starch.

### Results and discussion

Preliminary experiments were carried out to determine the optimum temperature at which starch hydrolysis could be stimulated in leaves as a result of application of a growth-regulating compound. One hundred forty-four young plants were selected and divided into six equal groups. Leaves of the plants in three of these lots were then sprayed with an emulsion containing 1 per cent indoleacetic acid (percentage calculated on basis of weight of lanolin used, 7). The remaining three groups were sprayed with pure lanolin emulsion. Each group of treated plants, together with an equal number of controls, was then placed in darkness in separate rooms. The temperature of one room was controlled at  $62^{\circ}$ – $64^{\circ}$ , another at  $74^{\circ}$ – $76^{\circ}$ , and the third at  $90^{\circ}$ – $92^{\circ}$  F. By means of iodine tests the starch contents of control and treated leaves kept at the different temperatures were compared at intervals during the following 12-hour period of darkness. Neither treated nor control leaves hydrolyzed an appreciable amount of starch at the low temperature. Treated leaves at  $74^{\circ}$ – $76^{\circ}$  F. gave a negative starch test at the end of 12 hours of darkness, while control leaves kept at the same temperature showed a positive test. Approximately all the starch was hydrolyzed in both treated and control leaves kept at  $90^{\circ}$ – $92^{\circ}$  F. for 12 hours, and at no time during the experiment was there a noticeable difference between the amount of starch in treated as compared with control leaves kept at this temperature. Evidently the application of indoleacetic acid failed to stimulate the relatively slow diastatic activity in leaves maintained at a low temperature, nor was there a noticeable increase in the rapid rate of starch digestion in leaves kept at a high temperature; but its use did result in a definite increase in the diastatic activity in leaves kept at the moderate temperature of approximately  $75^{\circ}$  F. Subsequent experiments concerning the effect of this

and other growth-regulating compounds on diastatic activity were therefore carried out with a controlled temperature of 74°–78° F.

Before studying quantitatively the effectiveness of different growth-regulating chemicals on the rate of starch digestion, the optimum concentration of each substance to be used was determined. Emulsions were prepared which contained the substances to be studied in concentrations of 0.01, 0.1, 1, and 5 per cent of the weight of lanolin in the emulsion. Leaves were then treated with these emulsions, the plants placed in darkness, and the rate of starch hydrolysis determined by means of iodine tests made at intervals during a period of 12 hours. Optimum effects without apparent injury were observed when 1 per cent emulsions were used, with the exception of phenylacetic acid, in which case 0.1 per cent was most effective. As a result of these tests these optimum concentrations were used in all subsequent experiments.

In order to study the effect of various growth-regulating chemicals on starch digestion in immature leaves, a number of young plants which had accumulated a relatively small amount of reserve carbohydrates were selected and divided into nine equal groups. The plants of one lot were treated with pure lanolin and the leaves were immediately harvested, so that the starch content of representative leaves at the beginning of the experiment could be determined. Plants of another group were also treated with pure emulsion but harvested at the conclusion of the experiment as final controls. The remaining groups were treated individually with indoleacetic, naphthaleneacetic, indolepropionic, phenylacetic, indolebutyric, and naphthoxyacetic acids, and naphthalene acetamide. The plants were then kept in darkness for 12 hours, after which time the leaves were harvested and analyzed for starch.

This experiment was repeated in identically the same way except that older, more mature plants, which had accumulated a relatively large amount of reserve carbohydrate in the form of starch, were used. Diastatic activity in the relatively mature leaves sprayed with pure lanolin emulsion was not appreciably different from that of young rapidly growing leaves treated in the same way. This is evidenced by the fact that 64 per cent of the starch and dextrin was digested in the leaves of the former and 66 per cent in the leaves of the latter during a 12-hour period of darkness (table 1). However, the rate of starch digestion in the less mature leaves, which initially contained 9.7 per cent starch on the basis of solid matter, was definitely increased as the result of treatment with emulsions containing indoleacetic, naphthaleneacetic, indolepropionic, indolebutyric, and naphthoxyacetic acids. It was only slightly stimulated as a result of treatment with phenylacetic acid, while an emulsion containing naphthalene acetamide was ineffective under the conditions of this experiment.

When older leaves—which initially contained 20.4 per cent starch—were used,

application of the chemicals, with the exception of indoleacetic acid, resulted in a decrease in the rate of starch digestion during the 12-hour period of darkness immediately following treatment. The rate of starch digestion in the older leaves treated with indoleacetic acid was not appreciably different from that of control leaves.

Repeated experiments were carried out with leaves of young plants that were approximately 15 days old and which contained a relatively small amount of reserve carbohydrate in the form of starch, and also with mature leaves of plants approximately 30 days old which contained a larger amount of reserve carbohydrate. Data obtained substantiated the results of the former experiments, in that starch digestion was increased in the immature leaves as the result of treatment

TABLE 1

STARCH HYDROLYSIS IN RELATIVELY YOUNG AS COMPARED WITH MATURE BEAN LEAVES  
FOLLOWING APPLICATIONS OF GROWTH-REGULATING SUBSTANCES

TREATMENT	PERCENTAGE OF INITIAL STARCH CONTENT HYDRO- LYZED DURING 12-HOUR PERIOD OF DARKNESS		TREATMENT	PERCENTAGE OF INITIAL STARCH CONTENT HYDRO- LYZED DURING 12-HOUR PERIOD OF DARKNESS	
	YOUNG LEAVES	MATURE LEAVES		YOUNG LEAVES	MATURE LEAVES
Untreated. ....	.....	62	Indolepropionic acid. ....	86	44
Pure lanolin. ....	66	64	Naphthoxyacetic acid. ...	87	48
Naphthalene acetamide. ...	66	49	Naphthaleneacetic acid. .	88	56
Phenylacetic acid. ....	71	42	Indolebutyric acid. ....	90	59
Indoleacetic acid. ....	85	66			

and was slightly retarded in the mature leaves during a period of 12-24 hours of darkness. The leaves of the more mature plants failed to show epinastic responses, although those of the younger plants became curled and bent within 1 hour following treatment with emulsions containing indoleacetic, indolepropionic, naphthaleneacetic, and indolebutyric acids.

It is evident that the application of a number of different growth-regulating compounds increased the rate of starch digestion in young immature leaves. Applied to older, more mature leaves under the same conditions, these chemicals retarded diastatic activity. It would appear from these results that the effect of these chemicals on the rate of starch digestion was controlled to some extent by factors or conditions which varied with the relative maturity of the plant.

In previously reported experiments (7) using naphthaleneacetic acid, there was associated with starch digestion a marked but temporary increase in the percentage of sugar present in leaves kept in darkness. In the present investigation ex-



periments were carried out to determine whether leaves treated with indoleacetic acid gave a similar response. A number of selected plants were treated with an emulsion containing 1 per cent indoleacetic acid and placed in darkness, together with controls which were treated with pure lanolin emulsion. Leaves were collected at intervals during the following 15-hour period and analyzed for total sugars. The percentage of sugar in leaves treated with indoleacetic acid increased to twice that of control leaves within a period of 4 hours after treatment (table 2) and remained at a relatively high level for several hours. At the end of 15 hours of darkness, the sugar content of treated leaves had decreased so that it was again ap-

TABLE 2  
SUGAR CONTENT OF LEAVES SPRAYED WITH INDOLEACETIC ACID  
COMPARED WITH THAT OF CONTROL LEAVES

HOURS AFTER TREATMENT	AVERAGES OF DUPLICATE DETERMINATIONS OF PERCENTAGE SUGAR (DRY WEIGHT)		HOURS AFTER TREATMENT	AVERAGES OF DUPLICATE DETERMINATIONS OF PERCENTAGE SUGAR (DRY WEIGHT)	
	CONTROL	TREATED		CONTROL	TREATED
0*.....	2.6	2.6	11.....	3.3	4.5
4.....	2.4	4.9	13.....	1.9	3.0
7.....	2.9	4.3	15.....	1.7	1.9
9.....	2.6	4.5			

\* Sampled at time of treatment.

proximately equal to that of control leaves, and at the same time the starch content of treated leaves decreased to less than 1 per cent of the dry weight.

These results demonstrate that the treatment of leaves with indoleacetic acid resulted in a marked increase in their sugar content, during a period of darkness, as had already been observed in leaves treated with naphthaleneacetic acid. It is not known whether this increase in sugar in the leaf resulted from carbohydrate transported from other parts of the plant, or whether it resulted from the hydrolysis of more complex carbohydrates.

### Summary

1. Lanolin emulsions containing various growth-regulating chemicals were sprayed on attached bean leaves and the plants were subsequently placed in darkness, together with controls. Quantitative analysis of the starch and dextrin content of the leaves showed that application of sprays containing indoleacetic, naphthaleneacetic, indolebutyric, indolepropionic, and naphthoxyacetic acids resulted in marked increase in the rate of starch digestion. Phenylacetic acid resulted in only a slight increase, while naphthalene acetamide had no noticeable effect.

2. Treatment of relatively old and mature leaves with these various chemicals either failed to stimulate, or inhibited to some extent, the hydrolysis of starch during a 12-hour period of darkness following treatment.

3. Application of these growth-regulating chemicals did not result in a noticeable increase in the rate of starch digestion in leaves kept at 62°–64° or at 90°–92°, but was effective at 74°–76° F.

4. The sugar content of leaves kept in darkness increased appreciably for a period of time following treatment with indoleacetic acid, a response previously observed in leaves treated with naphthaleneacetic acid.

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# ONTOGENY OF MEDULLARY BUNDLES IN *APIUM GRAVEOLENS*

CONTRIBUTIONS FROM THE HULL BOTANICAL LABORATORY 519

EDITH C. LAMBETH

(WITH TWELVE FIGURES)

## Introduction

The occurrence of medullary bundles has been reported (5) for many species of the Umbelliferae, but there seems to be no uniformity in the number, arrangement, or differentiation of the bundles. NESTEL (4) mentioned the presence of small, isolated conducting bundles in the pith, proximal to the common vascular ring; he also observed one such bundle in the cortex. According to his description, they are inclosed in a lignified sheath of bast (libriform) wood parenchyma. The phloem is well developed while the xylem is limited, consisting of small vessels. This and other general references state that medullary bundles are found in many of the Umbelliferae, but the writer has found no report as to the origin and development of these anomalous structures. The present study was undertaken to determine the ontogeny of the medullary bundles in the flower stalk.

## Material and methods

Two varieties of celery (*Apium graveolens* L.) were used, the White Plume and the Golden Self Blanching. The plants were grown in the greenhouse and were harvested at different stages during flowering. Navashin's solution and formalin-alcohol-acetic were employed as fixing agents. Best results were obtained with material treated by a tertiary butyl alcohol series, rather than by xylol or chloroform. All sections were cut on a rotary microtome at 10  $\mu$ . Star razor blades, clamped in a Spencer razor-blade holder, worked satisfactorily for sectioning. A modification of the triple stain was used.

## Observations

The medullary bundles tend to be developed opposite the protoxylem points of the common vascular bundles (1). The earliest stage in their ontogeny is indicated by meristematic activity in the parenchymatous cells near the periphery of the pith (fig. 3). From the cells thus derived, an oil duct is formed as a schizogenous, intercellular space. Those cells, generally three (fig. 2), bordering directly on the duct are thin walled and epithelial in nature (2). Anticlinal divisions may

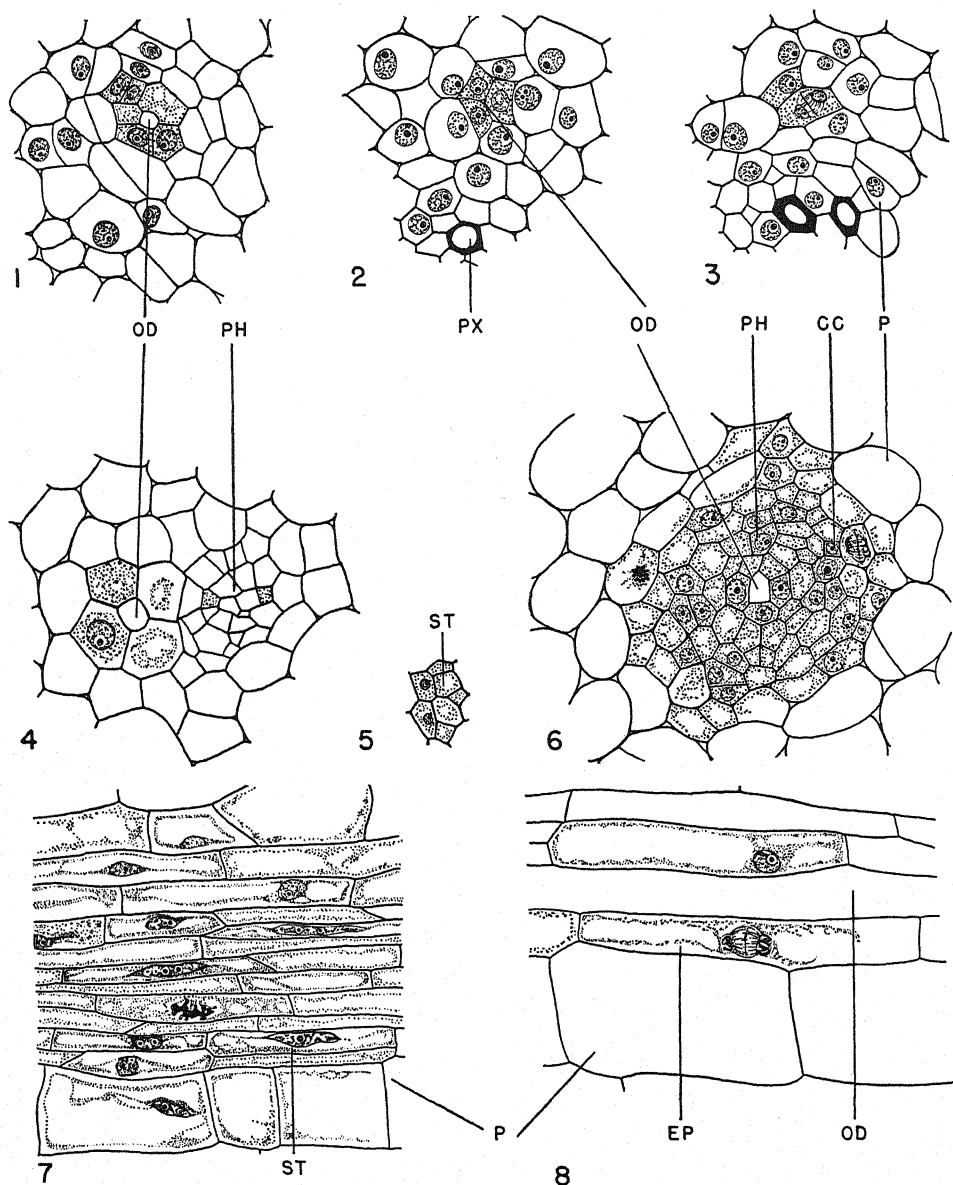
ultimately result in the formation of four to six epithelial cells, as shown in transverse view (fig. 1). Elongation of the duct and surrounding cells takes place by means of transverse divisions of the component cells (fig. 8).

Coincident with the differentiation of the epithelial cells, divisions also occur in adjacent parenchymatous cells (fig. 6). Characteristic of meristematic regions, these cells show prominent nuclei and darkly stained cytoplasm. Anticlinal, periclinal, and transverse divisions occur in rapid succession, and the derivatives differentiate as phloem. Development of the phloem in the medullary bundles parallels that of the phloem in the vascular bundles of the petiole (2). A parenchymatous cell undergoes several divisions, the second before the last giving rise to a phloem parenchyma cell and a sieve tube-companion mother cell. The sieve tube-companion mother cell divides, and the larger of the two resulting cells is the sieve tube (fig. 5). The smaller cell may give rise to one or more companion cells by transverse division. Figure 7 shows a phloem element dividing tangentially. Essentially the same stage of development is shown in transverse section in figure 6.

Although earlier divisions are not uniform on all sides of the epithelial cells (fig. 4), meristematic activity is established so that phloem finally surrounds the entire oil duct. Thus one oil duct generally becomes central in position. Frequently two ducts are differentiated so close together that phloem may become differentiated in the parenchyma that originally separated them, thus producing a bundle having two oil ducts. As many as five have been observed within a single bundle. Exceptionally, a duct was found situated adjacent to a row of cambium cells which had already differentiated xylem on the periphery.

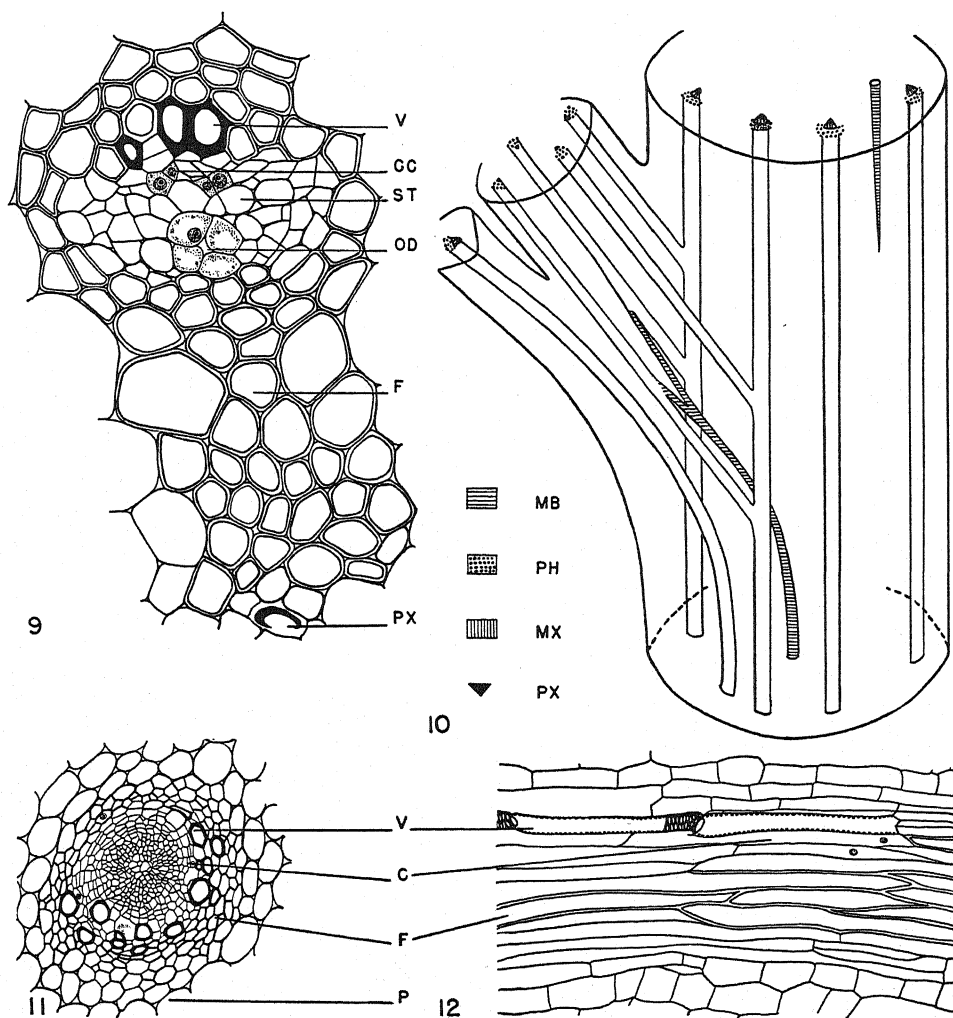
As the stem matures the active parenchymatous cells surrounding the phloem cylinder differentiate as a ring of cambium cells, two to four cells in thickness. From the derivatives of the cambium, secondary xylem and additional phloem elements are differentiated. The secondary xylem consists primarily of fibers, but in most bundles vessels occur scattered irregularly among the fibers, or arranged in discontinuous rows (fig. 11). Tapering ends and thick walls, with very small but abundant pits, characterize the fibers. Secondary wall thickening of the xylem elements is primarily of the scalariform type. Some reticulate thickenings were observed. In figure 12 the vessel is cut at such an angle as to show the disintegrated end wall (3).

The bundles in very mature stems are characterized by a greater development of fibers. This condition is brought about by the maturation of cambial derivatives into fibers which border directly on the phloem elements. In some cases a single row of cambium cells may remain. Frequently the parenchyma cells separating a medullary bundle and a common bundle may differentiate as fibers until direct contact is established between the two bundles (fig. 9). Many bundles were ob-



FIGS. 1-8.\*—Figs. 1-6, transsections showing stages in development of medullary bundle: 1, divisions in adjacent parenchyma cells; 2, oil duct surrounded by three epithelial cells; 3, parenchymatous cells opposite protoxylem point, dividing (figs. 1-3 from same bundle at different levels); 4, phloem development prominent on one side; 5, phloem detail; 6, phloem elements surrounding oil duct and divisions apparent on periphery. Fig. 7, longisection through a young bundle showing phloem element dividing tangentially. Fig. 8, same through oil duct showing epithelial cell dividing.

\* Abbreviations for figures: *c*, cambium; *cc*, companion cell; *ep*, epithelial cell; *f*, fiber; *mb*, medullary bundle; *mx*, metaxylem; *od*, oil duct; *st*, sieve tube; *v*, vessel; *px*, protoxylem; *p*, parenchyma.



FIGS. 9-12.—Fig. 9, transection through mature medullary bundle fused with protoxylem point of common vascular bundle by means of xylem fibers differentiated from parenchyma cells that separated them. Fig. 10, diagram of sector through node near base of umbel, showing two medullary bundles in their relation to the common vascular system. Fig. 11, transection through mature medullary bundle. Fig. 12, longisection through mature medullary bundle.

served to arise *de novo* and terminate in the pith (fig. 10). The phloem at the center of the mature bundles often appears crushed, and in some cases the epithelial cells show signs of deterioration.

The medullary bundles are cauline in nature. Through the internodes they follow a straight course, but at the nodes they may be distributed in any one or more of several ways. Those bundles situated on the side opposite a lateral branch may follow a straight course through several internodes. A bundle situated on the side of the stem giving rise to a lateral branch, however, may show anastomosing with another medullary bundle, branching, or differentiation so close to the phloem of the common bundle that the elements composing it are in contact with the phloem of the common bundles of the lateral branch. This last possibility is illustrated in figure 10, which shows that the phloem of the medullary bundle differentiates in contact with the phloem of the vascular bundles situated on the abaxial side of the lateral branch.

Contrary to the more common derivation of medullary bundles in dicotyledons (6), the bundles in celery show no direct relation to the leaf traces. Generally the term "medullary bundle" is used to describe intraxylary or internal phloem. Various theories as to the derivation and phylogenetic significance of these bundles have been advanced. According to WORSDELL (7), "the morphological origin of this internal phloem bundle is from an amphivasal bundle, for this latter is the typical and primitive condition of medullary bundles wherever they may occur." As quoted by WORSDELL, LAMOUNETTE "concludes (not only for Cucurbitaceae, but for other orders investigated) that internal phloem is an abnormal formation due to the activity of certain cells of the central conjunctive parenchyma, or is the ulterior evolution of the axis." This mode of development of the medullary bundle is essentially in agreement with the findings of the present investigation, but the phylogenetic significance of such bundles remains in question.

### Summary

1. A parenchymatous cell or several cells opposite the protoxylem point of any vascular bundle become meristematic. From the cells thus derived, an oil duct is formed schizogenously.
2. Parenchymatous cells surrounding the epithelial cells of the oil duct become meristematic. Anticlinal, periclinal, and transverse divisions occur in rapid succession, and the derivatives differentiate as phloem.
3. Although earlier divisions are not uniform on all sides of the epithelial cells, such meristematic activity is usually established that phloem finally surrounds the entire oil duct. Later a cambium is differentiated from the active parenchymatous cells surrounding the phloem. From the derivatives of the cambium secondary xylem and additional phloem elements are differentiated.

4. The medullary bundles are cauline in nature. Initiation of these bundles may be *de novo* at any level of the stem and terminate at any level. Frequently, at the nodes, they differentiate so close to the phloem of the common bundle that the elements composing them are in contact with the phloem of the common bundles of a lateral branch.

5. There is no direct connection of the medullary bundles with the leaf traces.

Grateful acknowledgment is made to Dr. H. E. HAYWARD for suggesting this problem, and to Dr. E. J. KRAUS for helpful suggestions during the progress of the investigation.

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# ABSORPTION SPECTRA OF THE CAROTENOIDS IN THE RED AND BROWN FORMS OF A PHOTOSYNTHETIC BACTERIUM

C. S. FRENCH

(WITH ONE FIGURE)

## Introduction

*Streptococcus varians*, a species of purple bacteria capable of photosynthetic CO<sub>2</sub> reduction, if hydrogen is available, will when grown in the absence of O<sub>2</sub> form a deep brown culture in yeast extract medium. If air or O<sub>2</sub> is admitted during or after the growth period the culture becomes bright red in a few hours. The addition of oxidizing or reducing substances to a suspension of the cells or to the water extracts described later does not bring about this color change rapidly, nor has the red form been changed back to the brown. Water extracts of the cell juice from brown cells remain brown for days when in contact with air. It therefore seems likely that the brown to red change is an enzymatic process taking place only in the intact bacteria and possibly having some relation to cell respiration. These bacteria contain two types of pigments: green methyl alcohol-soluble bacteriochlorophyll (1, 9) and reddish acetone and fat solvent-soluble carotenoids (6, 10). Only the former is used as the light absorber for photosynthesis (3), while the function (if any) of the latter is, like the use of the carotenoids in green plants, still unknown. A knowledge of these spectral changes in photosynthetic bacteria may have a bearing on the general problem of carotenoid function. The absorption spectra reported here afford a basis for a chemical investigation of this phenomenon and in themselves allow some speculation as to its nature. Furthermore, since quantitative photochemical measurements of the assimilation of carbon dioxide and hydrogen by near infra-red radiation have been made with this species (2), it is of interest to know the absorption spectra of the different colored forms.

From these measurements it appears probable that the change from brown to red when O<sub>2</sub> is allowed to come in contact with anaerobically grown bacteria is due either to the formation of an extra pigment with an absorption band having a maximum at 550 mμ or to the modification of an existing carotenoid, so that it has an extra band at that place.

## Experimentation

In order to make precise spectral absorption measurements of the bacterial pigments in the cells, it is advantageous to have a clear solution of the pigments,

so that scattering of light by the cell walls can be avoided. Extraction by organic solvents changes the position of the absorption bands of leaf and photosynthetic bacteria pigments. This group of bacteria, however, like some green plants, contain their colored components attached to a water-soluble protein (7, 8, 9). This protein pigment complex, photosynthin, may be obtained in a clear and fairly stable solution by breaking open the cell walls of a suspension of bacteria (4, 5).

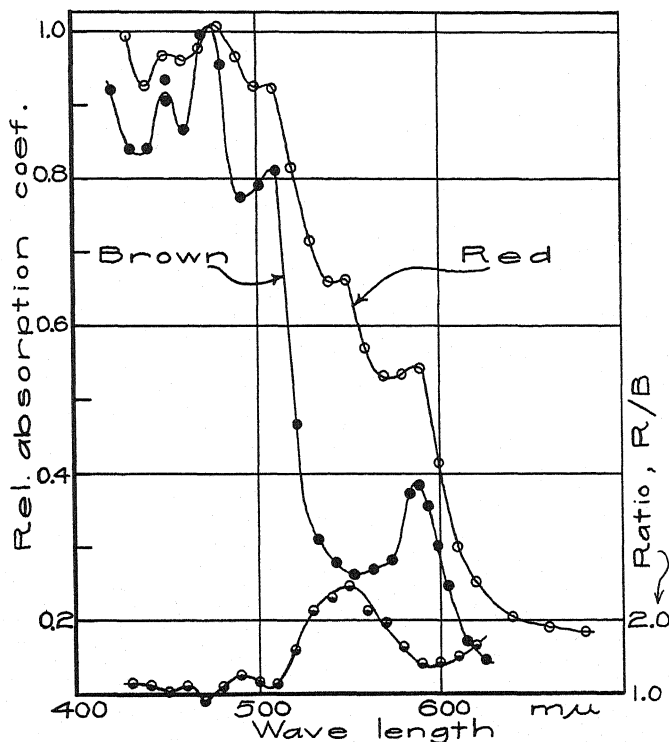


FIG. 1.—Absorption spectra of red (upper curve) and brown (lower curve) forms of *Streptococcus varians*. Ratio of curves shown at bottom of figure.

A thick suspension of *Streptococcus* (*Rhodopseudomonas* or *Phaeomonas*) *variens* (strain Original<sup>1</sup>) was put in a glass hypodermic syringe cylinder whose tip was sealed with de Khotinsky cement. The plunger was then inserted. The cylinder was held in the chuck of a lathe and rotated slowly while the plunger was gently forced in and kept from revolving by being fixed to the tail stock. The liquid suspension was squeezed out through the space between the well-fitting glass surfaces. A small percentage of the bacteria emerging were broken open and their

<sup>1</sup> This strain does not assimilate  $H_2$ , at least under conditions favorable for that reaction in the strain designated as Cr 1 by VAN NIEL, although it appears spectroscopically identical.

cell juice liberated into the surrounding liquid. This liquid was then centrifuged, the supernatant fluid diluted with water, and the absorption curve measured in the visible region with a Koenig-Martens spectrophotometer. Curves were made both for extracts of the brown bacteria grown as usual without air and the red bacteria from the same strain grown with air bubbling through the culture. As the percentage of cells broken by this treatment was not controllable, the curves are not comparable as to absolute height but are brought together at their maxima. Figure 1 shows these curves.

### Results

The bands at  $590\text{ m}\mu$  are due to bacteriochlorophyll and the others to the carotenoid pigments, as has been established by extraction experiments. The red bacteria show an extra absorption band at  $550\text{ m}\mu$  and less sharp bands of the carotenoid pigment. The apparent raising and blurring of the  $590\text{ m}\mu$  bacteriochlorophyll band is evidently due to the increased absorption in this region by part of the  $550\text{ m}\mu$  band. The bottom curve with half-filled circles is the ratio of the upper two curves and may be interpreted as the relative absorption curve of an extra pigment produced by the action of oxygen on the brown bacteria. It is possible, however, that some of the carotenoid pigment present in brown bacteria is altered by the action of  $\text{O}_2$ , making it absorb at longer wave lengths so that the effect might be attributed equally well to a modification of a pigment existing also in the brown type. This interpretation is favored by the blurring of the three carotenoid bands. So far no attempts have been made to separate this hypothetical extra pigment and study its chemical nature. Its absorption spectrum would suggest that it was not a carotenoid.

### Summary

1. Bacteria may be ground with a hypodermic syringe to liberate the water-soluble constituents.
2. Both aerobically grown (red) and anaerobically grown (brown) extracts of *Streptococcus varians* thus prepared have been examined spectroscopically.
3. An extra band at  $550\text{ m}\mu$  is found in the red form.

This work was done at the Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts.

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# DESMIDS OF MIRROR LAKE, UTAH

EDNA SNOW

Mirror Lake is one of many glacial lakes located in the Uinta Mountains of Utah. The water of the lake is acid, having a pH of about 5.1, according to TANNER (6). The collections of algae from this lake were made during August and

TABLE 1

SPECIES FOUND	No. OF COLLEC-TIONS	SPECIES FOUND	No. OF COLLEC-TIONS
Netrium digitus var. constrictum W. West.....	I	Cosmarium margaritiferum Menegh....	I
Penium spirostriolarum Barker.....	I	Cosmarium subspeciosum var. validium Nordst.....	I
Gonatozygon brebissonii De Bary.....	I	Cosmarium subturgidum fa. minor Schmidle.....	I
Closterium abruptum W. West.....	2	Cosmarium costatum Nordst. Fa.....	I
Closterium costatum Corda.....	2	Cosmarium subcucumis Schmidle.....	I
Closterium moniliferum (Bory) Ehr.....	2	Cosmarium hammeri var. protuberans W. & W.....	I
Closterium lanceolatum Kutz. var.....	I	Cosmarium subcrenatum var. isthmochondrum.....	I
Closterium lineatum Ehr.....	I	Staurostrum ophiura Lund.....	I
Closterium parvulum Naeg.....	I	Staurostrum grande Bulnh.....	I
Pleurotaenium ehrenbergii (Breb.) De Bary.....	I	Staurostrum natator var. crassum W. & W.....	I
Pleurotaenium coronatum var. nodulosum (Breb.) West.....	I	Staurostrum vestitum Ralfs.....	I
Pleurotaenium trabecula (Ehr.) Naeg..	I	Staurostrum polytrichum Perty.....	I
Pleurotaenium truncatum (Breb.) Naeg.	I	Staurostrum punctulatum Breb.....	I
Cosmarium crenatum Ralfs.....	I	Euastrum verrucosum var. rhomboideum Lund.....	4
Cosmarium bioculatum Breb.....	I	Euastrum gemmatum Breb.....	2
Cosmarium meneghinii Breb.....	I	Euastrum elegans (Breb.) Kutz.....	5
Cosmarium subcrenatum Fa.....	5	Euastrum bidentatum Naeg.....	2
Cosmarium turpinii Breb.....	I	Euastrum ansatum Ehr. var.....	2
Cosmarium turpinii var. exmium W. & W.....	2	Euastrum didelta (Turp.) Ralfs.....	2
Cosmarium angulosum var. concinnum W. & W.....	I	Sphaerosozma granulatum Roy et Biss..	I
Cosmarium margaritatum (Lund.) Roy et Biss.....	2	Hyalotheca mucosa (Mert.) Ehr.....	I
Cosmarium punctulatum var.....	I	Micrasterias americana (Ehr.) Ralfs....	I

September of 1937 and 1938. Algal forms were less abundant here than in many of the more shallow lakes, although they were plentiful near the north end, where a small stream of water enters. Desmids were the most common form of algae

collected. They were found associated with other green algae on the muddy bottom at depths of 4-12 feet.

Very little work has been done on the algae of Utah. HARRISON (1), NORRINGTON (2), TANNER (5), and SNOW (3) reported algae from various regions in the state. From the available lists, only six of the species of algae listed in table 1 have been previously reported from Utah. NORRINGTON reported *Pleurotaenium trabecula* (Ehr.) Naeg.; SNOW (4) reported *Closterium moniliferum* (Bory) Ehr., *Pleurothaenium threnbergii* (Breb.) De Bary., *Euastrum elegans* (Breb.) Kutz., and *Micrasterias americana* (Ehr.) Ralfs; and TANNER (5) reported *Euastrum verrucosum* Ehr. but not the variety *rhomboideum* Lund. included here.

Table 1 gives the species collected. The number following each form indicates the number of collections in which each species was found, roughly indicating the relative abundance of the species. Eleven genera and forty-four species were found. PRESCOTT reports *Stauroastrum natator* var. *crassum* W. & W. to be a new form. Identification of all species was made by G. W. PRESCOTT of Albion College, Michigan. The writer is greatly indebted to him and also to Dr. B. F. HARRISON, of Brigham Young University, for their help and encouragement.

DEPARTMENT OF BOTANY  
BRIGHAM YOUNG UNIVERSITY  
PROVO, UTAH

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## CURRENT LITERATURE

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*The New Systematics*. Edited by JULIAN HUXLEY. Oxford: Clarendon Press, 1940. Pp. viii+583. Figs. 57. \$6.00.

The relation of various biological sciences to taxonomy is discussed by various authors, mostly Englishmen. Chapters dealing mainly with botanical material include: Experimental and synthetic plant taxonomy, by W. B. TURRILL; Taxonomic species and genetic systems, by C. D. DARLINGTON; Ecological aspects of plant taxonomy, by E. J. SALISBURY; Taxonomic problems in fungi, by J. RAMSBOTTOM; Taxonomic botany, with special reference to the angiosperms, by T. A. SPRAGUE; Natural hybridization in relation to taxonomy, by H. H. ALLAN; Origin and behaviour of cultivated plants, by M. B. CRANE; and The new systematics of cultivated plants, by N. I. VAVILOV. J. S. HUXLEY brings the various fields, both zoological and botanical, together in a well-balanced introductory chapter.

The major factorial complexes involved in speciation include hereditary variation, isolation, and selection of natural populations. The action of these factors among cultivated plants shows the reciprocal principles involved in artificial and natural evolution. Both plants and animals are under fundamentally similar directive forces, the major difference being the gross chromosomal differences often associated with speciation in asexual, parthenogenetic, or self-fertilized organisms.

There is considerable difference among the species definitions by the various authors. When the evidence from all the correlated fields is summarized, however, it would seem that the species may be defined as that evolutionary stage at which a genetically distinctive, reproductively isolated, natural population becomes established. From both a theoretical and practical standpoint, such a definition conforms to the general practice of biologists working in various fields and fits the large number of species sufficiently well studied. Difficulties in the application of such a concept appear in the few species which show gradual hereditary change in complete paleontological series, and among the asexual populations where reproductive isolation is established with the origin of the individual and slight hereditary change occurs gradually, as in strains of bacteria.

The book is a landmark in progress toward collaboration of scientists from various divisions of biology. Although divergent views are often found among specialists, any attempt to formulate fundamental principles tends to bring these differences of opinion into the open, where they may be subjected to critical evaluation based upon evidence from many directions.—A. E. EMERSON.

*A Manual of Aquatic Plants*. By NORMAN C. FASSETT. New York: McGraw-Hill Co., 1940. Pp. vii+382. Illustrated. \$4.00.

Taxonomists have been very conservative in making the facts of their science readily available for the use of laymen or scientists untrained in systematic botany. A departure from this situation has become imperative in view of the increasing need for quick and accurate plant identification in the field by workers in the branches of wildlife, grazing, forestry, and soil conservation. Aquatic biologists, including experienced botanists, have reason to be grateful for the

present volume, which leads the field in this direction. A competent taxonomist has devoted much time and effort to compiling simple but accurate artificial keys, closely supplemented by original drawings illustrating all technical points, which facilitate quick identification of most macroscopic plants, whether sterile or flowering, normally germinating and growing with at least their bases in water. The territory covered ranges from Missouri and Minnesota to Virginia and the Gulf of St. Lawrence. The flora of bogs, small woodland brooks, waterfalls, and tidal, salt, and brackish waters is not included.

Part I, 33 pages, is a general key, based entirely on vegetative characters, which leads to the family, genus, or species name, and reference to the descriptive treatment in part II, 306 pages. This in turn is primarily a key to genera and species under each family, but in some cases necessarily requires the use of flower or fruit characters. Brief notes or descriptions are given for some families and genera, but most of the descriptive treatment of them, as well as of species, is given in the keys. A short statement of habitat and range follows most species' names. In spite of this brief textual treatment, there is rarely need for more adequate confirming description than is supplied by reference to the excellent and profuse illustrations, drawn with emphasis on the key characters. Families follow the ENGLER and PRANTL sequence, but genera may be grouped for the sake of convenience, as in the grasses, where they are placed in the order of their importance to the aquatic biologist. His interests are also considered in the appendix, which is a compilation, from cited papers, of the uses of aquatic plants by birds and mammals and of the relations of plants to fish.—C. E. OLMSTED.

*Die Methoden der Fermentforschung.* Leipzig: Georg Thieme, 1940.

This ambitious undertaking, which attempts to cover the field of enzyme research, is edited by EUGENE BAUMANN of Tübingen and KARL MYRBÄK of Stockholm. The prospectus indicates about 3500 pages, 800 figures, and reference to about 6000 titles. The work is deployed in three main sections, following the introduction: a general section which includes the substrates and enzymes; a special section which covers the hydrolases, desmolases, assimilation, antienzymes, and enzyme models; and a third section devoted to industrial and clinical problems involving enzyme research.

Only one part has been distributed. This part carries the first 172 pages and contains the following contributions: Simple synthetic substrates of the lipases, by EUGENE BAUMANN; The naturally occurring glycerides, by KARL HUGO BAUER; Acetylcholine, by ROBERT AMMON; Gallotannin and the simple substrates of tannase, by OTTO TH. SCHMIDT; Chlorophyll, by HANS FISCHER; Simple esters of phosphoric acid, by EBERHARD HACKENTHAL and MARIA KOBEL; Inositephosphoric acid, by THEODOR POSTERNAK; Phosphatides and their hydrolytic products, by SERAFINO BELFANTI, ALBERTO ERCOLI, and MARIA FRANNCIOLI; Simple esters of sulphuric acid, by HACKENTHAL and KOBEL; Biologically important carbohydrates and glycosides, by E. L. HIRST and L. PEAT; Extraction of natural heterosides, by J. RABATÉ; Synthetic glucosides, by HORST ELSNER; and part of a paper on thioglucosides, by FRITZ WREDE. The standing and ability of these authors guarantee the quality of the compilations and discussions. If conditions in Europe permit the completion of this cooperative venture, it should be an extremely valuable aid and stimulus to enzyme research throughout the world.

Subscribers are reminded that the publisher considers that an order placed for the first section implies that all will be accepted and paid for. The various sections cannot be sold separately. An impressive beginning has been made, and it is hoped that the entire work may become available.—C. A. SHULL.



*Bibliography of References to the Literature on the Minor Elements and Their Relation to Plant and Animal Nutrition. First Supplement* (pp. 24) and *Botanical Index* (pp. 82). 120 Broadway, New York: Chilean Nitrate Educational Bureau, Inc. 1940.

During recent years the Chilean Nitrate Educational Bureau has rendered an important service to nutritional research in the publication of three editions of this volume. The third edition, issued in 1939, listed more than 4600 references.

In a rapidly growing field it is out of the question to keep the bibliography up to date by reprinting the entire work at frequent intervals. To offset this difficulty, the Bureau has instituted the publication of supplements to the third edition. The first of these supplements contains 82 pages, including the indices. The references are listed by elements, in alphabetic order, a most convenient arrangement.

In addition to this service, a botanical index of the third edition has been prepared, which lists the plants in alphabetic order, together with all the nutrient elements which have been tested with each plant, listed alphabetically under each plant name, usually the common name. This renders the whole work more useful, and the volume becomes an indispensable tool for rapid information about any plant or any element. This feature is also carried over into the supplements. The first supplement has three sections to its index: first, by elements; second, a botanical index; and third, an author index.

The Chilean Nitrate Educational Bureau deserves the thanks of all workers in plant and animal nutrition for this valuable contribution to their research.—C. A. SHULL.

*Leaves and Stems from Fossil Forests.* By R. E. JANSSEN. Springfield: Illinois State Museum, Popular Science Series 1:1-190, 1939. Figs. 165. \$1.50.

*Some Fossil Plant Types of Illinois.* By R. E. JANSSEN. Springfield: Illinois State Museum, Scientific Papers 1:1-125, 1940. Pls. 28. \$1.25.

These publications describe, the first in nontechnical but accurate terms and the second more scientifically, some of the more common plant fossils from Illinois. So much advance has been made in our knowledge of fossil plants that it has seemed necessary to restudy the LESQUEREUX types in the Worthen collection of the Illinois State Museum, and this task has been undertaken by JANSSEN. Some fifty-nine species have been examined and only eighteen specific names remain as given by LESQUEREUX; two species have been specifically renamed and thirteen have been found valueless as specific types.

The second publication includes a description of ten new species and varieties of fossil plants recently discovered in the Mazon Creek region of Illinois.—G. D. FULLER.

*Elements of Botanical Microtechnique.* By JOHN E. SASS. New York: McGraw-Hill Co., 1940. Pp. ix+222. \$2.50.

This elementary manual is designed for the beginning student in the field of botanical microtechnique. Part I, which covers general principles and methods, takes up the common procedures in the selection and collection of suitable materials, killing and fixation for securing structural details, dehydration, infiltration with paraffin and celloidin, sectioning, staining of sections and whole mounts for permanent microscopic preparations. Part II deals with specific methods and gives more detailed directions for the various plant phyla. A special chapter is devoted to the construction, use, and care of the microscope, and another to photomicrography.—J. M. BEAL.

*Statistical Methods.* By GEORGE W. SNEDECOR. Ames, Iowa: Collegiate Press, 1940. Pp. xiii+422. Illustrated. \$3.75.

The third edition of this excellent work has been improved by correction of errors appearing in the second edition, by brief additions at the ends of chapters 6 and 16 (on linear regression and binomial and Poisson distributions), and by the addition of chapter 17, on design and analysis of samplings. This last chapter is a very practical one, taking up the problems of sampling from various types of populations, size of samples, etc. Actual examples are used to illustrate the methods.

This volume is the best current source of information for those who need to examine results for their validity.—C. A. SHULL.



# THE BOTANICAL GAZETTE

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## BIOCHEMICAL NITROGEN FIXATION STUDIES. III. PRODUCTION AND OXIDATION OF ETHYL ALCOHOL BY LEGUME NODULES<sup>1</sup>

C. A. LUDWIG, FRANKLIN E. ALLISON, SAM R. HOOVER,  
AND FRANCIS W. MINOR

(WITH ONE FIGURE)

### Introduction

It was shown in the first paper of this series (1) that when detached legume nodules are cultured for a short time (either with or without added glucose) under conditions allowing sufficient oxygenation of the tissues, a respiratory quotient approximating 1.00 is obtained. This respiratory quotient (R.Q.) corresponds to the complete oxidation of sugar. If the oxygenation is insufficient, however, the R.Q. is greater than 1.00 and becomes infinite in the limiting case where no molecular oxygen is available, since CO<sub>2</sub> is still given off. Such respiratory quotients correspond to the splitting off of CO<sub>2</sub> from the sugar, leaving as a residue one or more complex compounds. Since it is well known that ethyl alcohol is produced by most plant tissues under such conditions, it seemed desirable to determine to what extent, if any, it is also produced by nodules, and if so whether or not it is oxidized by them when the tissues are fully oxygenated. This paper gives the results obtained, and in addition certain results with legume and non-legume roots which were used for comparison.

### Methods

**WARBURG MANOMETRIC METHOD.**—The Warburg manometric procedure as used in the work with nodules has already been described (1<sup>2</sup>). Its suitability for the

<sup>1</sup> The writers are indebted to Dr. R. T. MILNER and Mrs. MILDRED S. SHERMAN for advice concerning the alcohol determinations, to Mrs. SHERMAN for most of the determinations, and to Dr. DEAN BURK for criticism of certain parts of the text.

<sup>2</sup> In the preceding two papers of this series (pp. 518 and 536), the symbols QO<sub>2</sub>, QO<sub>2</sub>, QO<sub>2</sub>(N), and R.Q. are defined erroneously in terms of ml. O<sub>2</sub> or CO<sub>2</sub> per mg. per hour, whereas the correct definition should have been expressed in terms of cu. mm. per mg. per hour.

present study depends chiefly on the fact that the R.Q. (0.67) corresponding to complete oxidation of alcohol is quite different from that (1.00) of nodules respiring aerobically without external substrate or with sugar. Therefore if varying quantities of alcohol be added to the nodules and it is oxidized, corresponding variations of the R.Q. can be expected, from a value of 1.00 where no alcohol is added, to 0.67 where enough is added to replace sugar entirely in respiration. The range of variation in R.Q. values obtained for nodules by this technique, as may be noted from the tables that follow, is greater than is usually observed with bacterial cultures, owing partly to greater difficulty in getting uniform samples and partly at least to the higher  $\text{CO}_2$  values of the blanks, and the resulting greater gross variation. While too much significance must not be given to the absolute magnitude of some of the results, they are in general clear cut and ample for the conclusions drawn.

CHEMICAL METHOD.—While the manometric method can be expected to give fairly conclusive results as to the oxidation of alcohol under aerobic conditions, the evidence is indirect; in any case it cannot do more than indicate the general nature (average molecular formula) of the residual compounds resulting from the fermentative splitting off of  $\text{CO}_2$  from sugar under anaerobic conditions. It seemed advisable, therefore, to determine chemically the accumulation or disappearance of alcohol under both anaerobic and aerobic conditions. For this purpose a micro adaptation of CLARK's modification (7) of the Vieböck and Schwappach method for the determination of methoxy and ethoxy groups was used. In principle the method depends upon the production of a volatile iodide by boiling the material in hydriodic acid. The iodide is distilled off, decomposed, and the resulting iodine determined volumetrically. The method seems to be practically specific for alcohol in plant tissues, because of all the groups (methoxy, ethoxy, isopropoxy, and cyclopropoxy) that will produce volatile iodides under the conditions named, only ethoxy can ordinarily be expected as a volatile compound in such tissues. Trial runs on pure alcohol solution gave recoveries within the errors of the independent information concerning its concentration. A slight failure of complete recovery is of negligible importance in a study such as this, since the controls and the experimental cultures are affected alike and the differences are affected very little, if at all. The alcohol was separated from the tissues and solution by distillation.

Nodules or roots of the following plant species were used: cowpea (*Vigna sinensis* (L.) Endl.), Korean lespedeza (*Lespedeza stipulacea* Maxim.), crotalaria (*Crotalaria spectabilis* Roth.), soybean (*Soja max* (L.) Piper), common white sweet clover (*Melilotus alba* Desr.), a yellow-flowered sweet clover (*M. dentata* (Waldst. and Kit.) Pers.), hairy vetch (*Vicia villosa* Roth.), common vetch (*V. sativa* L.), crown vetch (*Coronilla varia* L.), red clover (*Trifolium pratense* L.), oats

(*Avena sativa* L.), corn (*Zea mays* L.), and narrow-leaved plantain (*Plantago lanceolata* L.).

## Results

### ANAEROBIC PRODUCTION OF ALCOHOL BY NODULES

Data on the anaerobic production of alcohol by nodules are given in table 1. The experiments lasted about 2.5–4.7 hours and were conducted in special large vessels which allowed the CO<sub>2</sub> to be determined manometrically on samples large enough for the chemical determination of alcohol. The results are the means of two cultures except where specified otherwise, but the controls consisted of single lots of 10 gm. each of nodules. In calculating the CO<sub>2</sub> produced it was necessary

TABLE 1  
ALCOHOL AND CO<sub>2</sub> PRODUCTION BY NODULES UNDER ANAEROBIC CONDITIONS

EXP. NO.	PLANT	NODULES USED (GM.)	GLUCOSE ADDED (%)	GAS USED	CO <sub>2</sub> PRO- DUCED (MG.)	C <sub>2</sub> H <sub>5</sub> OH			EQUIVA- LENT TO CO <sub>2</sub> PRO- DUCED (MG.)
						AT START (MG.)	AT CLOSE (MG.)	PRO- DUCED (MG.)	
44.....	Cowpea	5	2.00	N <sub>2</sub>	4.27	0.60	4.17	3.57	4.47
45.....	Soybean	5	1.14	N <sub>2</sub>	10.16	1.04	9.88	8.84	10.63
45.....	Soybean*	5	0.00	N <sub>2</sub>	7.02	1.04	7.43	6.39	7.35

\* One culture only.

to include that produced after the vessels were filled with nitrogen and before the first readings were taken. This amount was estimated on the assumption that production of CO<sub>2</sub> during the interval before the readings were begun was at the same rate at which it occurred afterward. The last column of the table records the amount of alcohol that would have been produced to correspond to the CO<sub>2</sub> found if hexose were the substrate affected and if only CO<sub>2</sub> and alcohol were produced by its breakdown. The values are included to give an idea of the relative abundance of alcohol and of other products of fermentation produced by the nodules.

The following points seem to be fairly well established by the data in the table: (1) alcohol is probably always present in small amounts in the nodules under usual conditions; (2) alcohol accumulates under anaerobic conditions; and (3) assuming that hexose is the substance broken down to CO<sub>2</sub> and unoxidized compounds, alcohol is by far the most abundant of the latter, averaging in these experiments about 83 per cent of them. These results held whether or not glucose was added in the medium, but in its presence the quantity of alcohol produced may have been increased to some extent.

## AEROBIC OXIDATION OF ALCOHOL BY NODULES

The aerobic oxidation of alcohol was investigated by both manometric and direct chemical methods. Table 2 shows the effect on R.Q. and on  $QO_2$  of the addition of different amounts of alcohol to the medium, and table 3 records the effect of glucose and alcohol combined. Figure 1 shows the means of the R.Q.'s for the five experiments with cowpea nodules recorded in table 2. The effect of greater amounts of alcohol than 5 per cent was investigated in only one experiment, because in this instance 10 per cent appeared to be toxic, as evidenced by a considerable reduction of oxygen uptake ( $QO_2$ ).

Table 2 and figure 1 show that a progressively smaller decrease in R.Q. resulted from each increase in alcohol concentration, until the R.Q. was reduced almost to that (0.67) characteristic of the complete oxidation of alcohol.

The last two lines in table 2 show the relative value of the  $QO_2$  with various percentages of alcohol, together with the number of experiments on which each value is based. Below the toxic range of the material the  $QO_2$  is increased by a mean maximum of about 5 per cent or 6 per cent, as compared with about 12 per cent for glucose. It does not follow, however, that only 5 per cent or 6 per cent of the oxygen consumed is used in the oxidation of alcohol, for if such were the case the R.Q. would not be reduced below 0.98. That it actually approached 0.67 with little change in  $QO_2$  suggests rather strongly that the alcohol reduced the amount of other substrate oxidized.

The results in table 3 show that the effects of alcohol and of glucose on the R.Q. are exerted in the presence as well as in the absence of the other, the final R.Q. when both are added being intermediate between that resulting from alcohol alone and that from glucose alone. The R.Q. values greater than 1.00 observed in the controls and glucose-only cultures indicate that, in spite of the use of practically pure oxygen as the aerating material, complete aerobiosis was not attained—except possibly with the *crotalaria* nodules. Indeed, with these nodules the values were abnormally low throughout. The table shows also that both substrates increased the  $QO_2$  in the presence as well as in the absence of the other, the increase when both were added being not much more than the sum of the increases produced by adding each alone. It is probable that this relationship would not hold if each were present in the concentration at which it produces its maximum effect.

These data show that alcohol and glucose are both acceptable substrates for oxidation by the nodules, and that each substance competitively inhibits the oxidation of the other. Furthermore, the fact that very small concentrations of alcohol reduce the R.Q.'s markedly and do not change the  $QO_2$ 's in proportion constitutes strong evidence that it—rather than glucose—is the compound preferentially oxidized.

In order to check by direct methods the oxidizability of alcohol by nodules,

TABLE 2  
OXIDATION OF ADDED ALCOHOL BY NODULES: MANOMETRIC RESULTS

EXP. NO.	PLANT	GAS USED		CONTROL		PERCENTAGE ALCOHOL IN MEDIUM* (WITHOUT GLUCOSE)						
				WITH 1% GLU- COSE	WITH- OUT GLU- COSE	0.02	0.1	0.5	1	2	5	10
28.....	Korean lespe- deza	Air	R.Q.	1.37	1.17	.....	.....	0.94	.....	0.87	.....	.....
			QO <sub>2</sub>	1.80	1.69	.....	.....	1.77	.....	1.72	.....	.....
30.....	Korean lespe- deza	80% O <sub>2</sub> 20% N <sub>2</sub>	R.Q.	1.03	0.97	.....	.....	.....	.....	0.66	.....	.....
			QO <sub>2</sub>	5.13	4.48	.....	.....	.....	.....	4.70	.....	.....
31.....	Korean lespe- deza	Air	R.Q.	.....	1.26	.....	.....	1.05	.....	0.98	.....	.....
			QO <sub>2</sub>	.....	1.50	.....	.....	1.40	.....	1.66	.....	.....
		80% O <sub>2</sub> 20% N <sub>2</sub>	R.Q.	.....	1.01	0.93	0.86	0.80	.....	0.68	.....	0.50
			QO <sub>2</sub>	.....	4.22	4.46	3.98	4.17	.....	4.06	.....	2.65
36.....	White sweet clover	O <sub>2</sub>	R.Q.	0.95	1.03	.....	.....	.....	0.69	.....	.....	.....
			QO <sub>2</sub>	4.84	3.83	.....	.....	.....	4.13	.....	.....	.....
50.....	Yellow sweet clover	O <sub>2</sub>	R.Q.	1.09	1.06	1.01	0.94	0.96	0.96	0.86	0.75	.....
			QO <sub>2</sub>	6.61	6.03	6.11	5.97	6.06	5.02	5.77	5.61	.....
49.....	Cowpea	O <sub>2</sub>	R.Q.	1.04	1.03	0.98	0.81	0.69	0.64	0.71	0.67	.....
			QO <sub>2</sub>	2.79	2.45	2.21	2.74	3.06	3.03	3.01	2.86	.....
55.....	Cowpea	O <sub>2</sub>	R.Q.	1.00	1.03	0.92	0.84	0.81	0.72	0.65	0.65	.....
			QO <sub>2</sub>	3.76	3.91	3.89	3.69	3.94	3.81	3.65	3.76	.....
56.....	Cowpea	O <sub>2</sub>	R.Q.	1.11	1.05	1.00	0.84	0.77	0.74	0.73	0.75	.....
			QO <sub>2</sub>	3.88	3.24	3.56	3.55	3.91	3.64	3.65	3.60	.....
57.....	Cowpea	O <sub>2</sub>	R.Q.	1.06	1.10	1.04	0.86	0.80	0.71	0.74	0.66	.....
			QO <sub>2</sub>	4.33	3.74	3.98	4.12	4.22	4.35	4.16	3.94	.....
58.....	Cowpea	O <sub>2</sub>	R.Q.	0.95	0.98	0.92	0.83	0.77	0.62	0.65	0.75	.....
			QO <sub>2</sub>	5.81	5.47	5.72	5.95	5.80	5.60	6.12	5.82	.....
Mean of experiments with cowpea.....		O <sub>2</sub>	R.Q.	1.03	1.04	0.97	0.84	0.77	0.69	0.70	0.70	.....
			QO <sub>2</sub>	4.11	3.76	3.87	4.01	4.19	4.09	4.12	4.00	.....
Mean of all experiments available.....		No. of experi- ments		9	.....	7	7	9	7	15	6	1
		Relative QO <sub>2</sub>		112	100	103	103	106	103	106	103	63

\* Most of these results are based on one culture only, but most of the no-alcohol and 2 per cent-alcohol figures are the means from two cultures.



some experiments were conducted in which alcohol was added to the culture medium. The amount present in nodules and medium at the end of an incuba-

TABLE 3  
OXIDATION OF ADDED ALCOHOL BY NODULES IN PRESENCE OF  
ADDED GLUCOSE: MANOMETRIC RESULTS

EXP. NO.	PLANT	GAS USED		SUBSTRATE ADDITIONS			
				GLUCOSE 1%	NOTHING	GLUCOSE 1%, ALCOHOL 2%	ALCOHOL 2%
75.....	Cowpea	O <sub>2</sub>	R.Q. QO <sub>2</sub>	1.28 6.37	1.17 5.80	0.84 6.77	0.81 6.22
78.....	Crotalaria	O <sub>2</sub>	R.Q. QO <sub>2</sub>	1.04 7.95	0.95 6.19	0.75 7.84	0.56 6.21
86.....	Hairy vetch	O <sub>2</sub>	R.Q. QO <sub>2</sub>	1.21 8.20	1.11 8.16	1.04 9.15	0.97 9.53
	Mean....	O <sub>2</sub>	R.Q. QO <sub>2</sub>	1.18 7.51	1.08 6.72	0.87 7.92	0.78 7.32
			Relative QO <sub>2</sub>	112	100	118	109

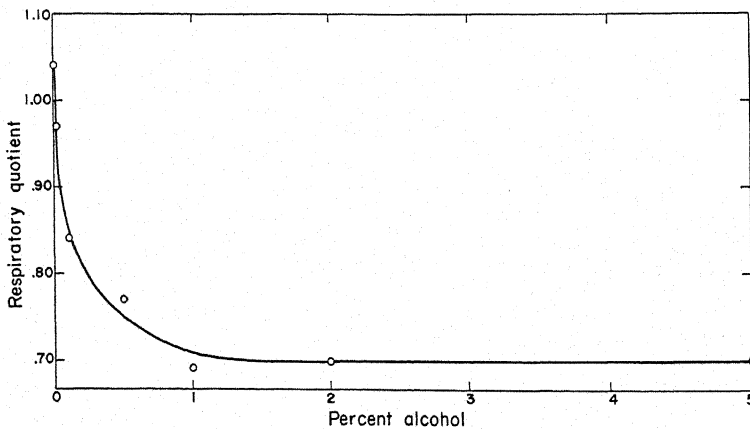


FIG. 1.—Effect of alcohol on respiratory quotient of cowpea nodules

tion period was then compared with that present at the beginning. For this purpose 3-gm. lots of nodules were placed with 5-cc. portions of 0.2 per cent alcohol in culture medium in tightly stoppered 125-cc. Erlenmeyer flasks in an atmosphere

of oxygen at room temperature, 24°–25.5° C., and shaken on a shaking machine during culture periods of 3–7 hours. Controls were distilled with as little delay as possible and without previous exposure to pure O<sub>2</sub>. It is believed that no significant error was introduced by the slight unavoidable delay in distilling the last of the controls, since a further control experiment showed little change in the alcohol content of nodules after several hours in air at room temperature. The results are given in table 4, which shows that the data of the chemical analyses agree with those from the manometric work in indicating that alcohol in culture medium is oxidized by legume nodules under suitable conditions.

TABLE 4  
OXIDATION OF ADDED ALCOHOL BY NODULES: CHEMICAL RESULTS

EXP. NO.	PLANT	GAS USED	DRY WT. NODULES (GM.)	C <sub>2</sub> H <sub>5</sub> OH		
				MEAN CONTENT OF CON- TROLS (MG.)	MEAN CON- TENT OF INCUBATED SAMPLES (MG.)	OXIDIZED PER GM. DRY MATTER PER HOUR (MG.)
60.....	Cowpea*†	O <sub>2</sub>	0.615	7.81	4.73‡	1.00
60.....	Cowpea†§	O <sub>2</sub>	0.615	7.09	4.66‡	0.79
61.....	Cowpea*	O <sub>2</sub>	0.534	7.21	1.91‡	1.99
61.....	Cowpea§¶	O <sub>2</sub>	0.534	6.50	2.93‡	1.34

\* Nodules crushed.

† Two controls and two experimental cultures.

‡ Calculated to 5 hours' duration.

§ Nodules not crushed.

|| One control and one experimental culture.

¶ Three controls and three experimental cultures.

#### ALCOHOL PRODUCTION AND OXIDATION BY PLANT ROOTS

It seemed reasonable to conclude from the work with nodules that they behave toward alcohol fundamentally like ordinary root tissues, since it is generally accepted that most plant tissues produce alcohol anaerobically. It was thought advisable, however, to check the point definitely by testing root tissues of various species. Small roots of both legumes and non-legumes were investigated for both alcohol production and alcohol consumption.

The roots were treated essentially in the same manner as the nodules, except that in the Erlenmeyer flask technique for detection of alcohol consumption the roots were occasionally shaken by hand instead of constantly by machine, since when machine shaking was attempted the roots of the culture aggregated into a thick clump in the middle of the vessel instead of remaining well distributed throughout the liquid. The duration of the experiments varied from 2.5 to 5.5 hours.

The results for alcohol production are given in table 5. It is clear from the data that, as already shown for nodules, ethyl alcohol and  $\text{CO}_2$  are produced in the small roots of both legumes and non-legumes under anaerobic conditions, and that the proportions of the two are roughly those to be expected if the main reaction concerned is a fermentative splitting of sugar into the two compounds. As a mean for the three kinds of roots used, the alcohol produced was about 85 per cent of the amount equivalent to the  $\text{CO}_2$  produced.

The results for the aerobic utilization of alcohol by roots secured by the manometric method are given in table 6 and those by the analytical method for experiments of about 3-4.5 hours' duration in table 7. They show that roots also

TABLE 5  
ALCOHOL AND  $\text{CO}_2$  PRODUCTION BY ROOTS UNDER ANAEROBIC CONDITIONS

EXP. NO.	PLANT	ROOTS USED (GM.)	GLUCOSE ADDED (%)	GAS USED	$\text{CO}_2$ PRODUCED (MG.)	$\text{C}_2\text{H}_5\text{OH}$			
						AT START (MG.)	AT CLOSE (MG.)	PRODUCED (MG.)	EQUIVALENT TO $\text{CO}_2$ PRODUCED (MG.)
44.....	Cowpea*	5	2.00	$\text{N}_2$	5.92	1.30	6.25	4.95	6.20
45.....	Soybean*	5	1.14	$\text{N}_2$	7.07	0.76	6.98	6.22	7.40
47.....	Corn†	4	1.00	$\text{N}_2$	3.61	0.31	3.80	3.49	3.78

\* One culture and one (10 gm.) control.

† Two cultures and two (8 gm.) controls.

oxidized alcohol aerobically. Thus legume and non-legume roots behaved alike in this respect. They show further that the  $\text{QO}_2$  was increased by alcohol in both legume and non-legume roots, but that—as with nodules—the increase was not great enough to account for the change in R.Q. It is likely that the alcohol reduced the amount of other substrate oxidized. One point of difference between legume nodules and roots, previously mentioned (2), is that the roots consistently gave a lower R.Q. than the nodules, the R.Q. for roots being less than 1.00 under aerobic conditions. This may mean (1) that the food reserves in the small roots are normally so low that they are quickly exhausted, with consequent oxidation of nitrogenous compounds; (2) that they include considerable amounts of some compound (possibly alcohol) other than sugar and which on complete oxidation has an R.Q. considerably less than unity; or (3) that incomplete oxidation of sugar occurs, with the formation of compounds with R.Q. values greater than 1.00. As will appear later, the evidence seems to favor the second of these possibilities.

TABLE 6  
OXIDATION OF ADDED ALCOHOL BY ROOTS: MANOMETRIC RESULTS

EXP. NO.	PLANT	GAS USED		CONTROL		PERCENTAGE ALCOHOL IN MEDIUM* (WITHOUT GLUCOSE)						
				WITH 1% GLU- COSE	WITH- OUT GLU- COSE	0.02	0.1	0.5	1	2	5	10
32.....	Korean lespedeza	Air	R.Q. QO <sub>2</sub>	0.97 2.35	0.80 1.50	.....	.....	.....	.....	0.73 1.93	.....	.....
		80% O <sub>2</sub> 20% N <sub>2</sub>	R.Q. QO <sub>2</sub>	0.94 3.01	0.68 2.06	.....	.....	.....	.....	0.58 2.09	.....	.....
33.....	Korean lespedeza	Air	R.Q. QO <sub>2</sub>	..... .....	0.85 1.64	.....	.....	.....	.....	0.65 1.59	.....	.....
		80% O <sub>2</sub> 20% N <sub>2</sub>	R.Q. QO <sub>2</sub>	..... .....	0.79 1.60	0.73 1.89	0.69 1.85	0.65 1.89	0.66 1.67	0.69 1.92	.....	0.69 0.59
53.....	Oat	O <sub>2</sub>	R.Q. QO <sub>2</sub>	0.96 2.05	0.92 1.75	0.87 1.73	0.86 1.70	0.81 1.62	0.80 1.68	0.79 1.53	0.66 1.16	.....
54.....	Oat	O <sub>2</sub>	R.Q. QO <sub>2</sub>	0.90 2.19	0.92 1.94	0.81 2.07	0.82 2.01	.....	.....	0.75 2.06	0.80 1.99	.....
Mean of experiments with oat .....		O <sub>2</sub>	R.Q. QO <sub>2</sub>	0.93 2.12	0.92 1.85	0.84 1.90	0.84 1.86	.....	.....	0.77 1.80	0.73 1.58	.....
Mean with Korean lespe- deza .....		Relative	QO <sub>2</sub>	151	100	118	116	118	104	111	.....	037
Mean with oat .....		Relative	QO <sub>2</sub>	115	100	103	101	93	96	97	85	.....
Mean with both species..		Relative	QO <sub>2</sub>	132	100	108	105	105	100	106	85	.....

\* Most of the results are from one culture only, but most of the no-alcohol and 2 per cent-alcohol figures are the means from two cultures.

TABLE 7  
OXIDATION OF ADDED ALCOHOL BY ROOTS: CHEMICAL RESULTS

EXP. NO.	PLANT	GAS USED	DRY WT. ROOTS (GM.)	C <sub>2</sub> H <sub>5</sub> OH*		
				MEAN CON- TENT OF CONTROLS (MG.)	MEAN CON- TENT OF IN- CUBATED SAMPLES (MG.)	OXIDIZED PER GM. DRY MATTER PER HOUR (MG.)
62.....	Cowpea	O <sub>2</sub>	0.394	13.10	12.55†	0.28
63.....	Red clover	O <sub>2</sub>	0.410	13.83	12.23†	0.78
64.....	Narrow-leaved plantain	O <sub>2</sub>	0.453	6.84	4.63†	0.98

\* Means of four controls and four experimental cultures.

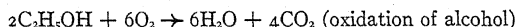
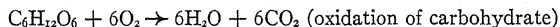
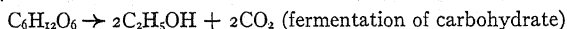
† Calculated to 5 hours' duration.

## EFFECT OF OXYGEN ON CONSUMPTION OF RESPIRABLE CARBOHYDRATE

If it be admitted, as the data already given strongly indicate, that the chief compounds concerned in fermentation and respiration of plant tissues are carbohydrate and alcohol, it is possible to calculate from the  $\text{CO}_2$  and  $\text{O}_2$  data certain functions which throw light on the processes concerned. In tables 8 and 9 certain such functions<sup>3</sup> are listed to show the effect of varying the oxygen concentration on the respiration of nodules and roots with different amounts of added glucose and alcohol.

It will be noted that with all the tissues used and with or without added alcohol or glucose, both  $\text{CO}_2$  production and  $\text{O}_2$  consumption were increased by increased

<sup>3</sup> The formulas for the calculation of these functions depend for fermentation and oxidation on the following equations:



The formulas are derived as follows: Following a common convention, a unit of carbohydrate is the amount which will produce a unit (in this instance 1 cu. mm. at standard temperature and pressure) of  $\text{CO}_2$  by fermentation. It is also the amount which will produce three units (3 cu. mm.) of  $\text{CO}_2$  by oxidation, since—as shown by the first two equations—on a molal or volume basis three times as much  $\text{CO}_2$  is produced by the oxidation of carbohydrate as by its fermentation. In oxidation of carbohydrate, the  $\text{CO}_2$  evolved and  $\text{O}_2$  consumed are equal and therefore interchangeable in calculation.

A unit of alcohol is the quantity produced by the fermentation of a unit of carbohydrate; therefore alcohol accumulated (per hour) equals  $\text{QCO}_2 - \text{QO}_2$ . This follows because it equals the total  $\text{CO}_2$  produced minus the  $\text{CO}_2$  of oxidation, and the  $\text{CO}_2$  of oxidation equals the  $\text{O}_2$  consumed. When carbohydrate is the substrate the value for alcohol accumulated is positive and is the same as that for carbohydrate fermented but not metabolized further; when alcohol is the substrate the value of course is negative.

Similarly carbohydrate consumed equals  $\text{QCO}_2 - \frac{1}{3}\text{QO}_2$ , for where carbohydrate is the substrate it equals the carbohydrate fermented to alcohol ( $\text{QCO}_2 - \text{QO}_2$ ) plus that oxidized ( $\frac{1}{3}\text{QO}_2$ ); and where alcohol is present also and being oxidized the formula remains the same since the  $\text{CO}_2$  produced is two-thirds of the  $\text{O}_2$  consumed.

Ratio of  $\text{CO}_2$  evolved under any condition of aeration to  $\text{CO}_2$  evolved anaerobically is evidently  $\frac{\text{QCO}_2}{\text{QCO}_2^{\text{N}_2}}$ . This is the reciprocal of the  $\frac{\text{I}}{\text{N}}$  or  $\frac{\text{NR}}{\text{OR}}$  ratio sometimes mentioned in work on plant respiration.

The ratio as used here, however, seems to serve the purpose of the present study better than the more common form.

Decrease (due to oxygen) in alcohol accumulated equals  $\text{QCO}_2^{\text{N}_2} - \text{QCO}_2 + \text{QO}_2$ ; that is, it equals alcohol accumulated (or carbohydrate fermented) anaerobically ( $\text{QCO}_2^{\text{N}_2}$ ) minus that ( $\text{QCO}_2 - \text{QO}_2$ ) accumulated at the oxygen concentration concerned.

Decrease (per unit of substrate oxidized) in alcohol accumulated equals  $\frac{\text{QCO}_2^{\text{N}_2} - \text{QCO}_2 + \text{QO}_2}{\frac{1}{3}\text{QO}_2}$ , for it obviously equals the decrease, due to oxygen, in alcohol accumulated ( $\text{QCO}_2^{\text{N}_2} - \text{QCO}_2 + \text{QO}_2$ ) divided by substrate oxidized ( $\frac{1}{3}\text{QO}_2$ ). The substrate oxidized may be either carbohydrate, alcohol, or a mixture of the two.

It should be mentioned that, by the use of these terms and functions, nothing is implied concerning the nature of the first step in normal respiration, or, in general, the mechanism or path of the process.

TABLE 8  
EFFECT OF OXYGEN CONCENTRATION ON RESPIRATION OF LEGUME NODULES

1	2	3	4	5	6	7	8	9	10	11	12
EXP. NO.	PLANT	SUBSTRATE ADDED	GAS USED	CO <sub>2</sub> EVOLVED	O <sub>2</sub> CONSUMED	RESPIRATORY QUOTIENT	ALCOHOL ACCUMULATED	CARBOHYDRATE CONSUMED	RATIO OF CO <sub>2</sub> EVOLVED TO CO <sub>2</sub> EVOLVED ANAEROBICALLY	DECREASE DUE TO OXYGEN, IN ALCOHOL ACCUMULATED	DECREASE PER UNIT OF SUBSTRATE OXIDIZED IN ALCOHOL ACCUMULATED
				Q <sub>CO<sub>2</sub></sub>	Q <sub>O<sub>2</sub></sub>	$\frac{Q_{CO_2}}{Q_{O_2}}$	Q <sub>CO<sub>2</sub></sub> - Q <sub>O<sub>2</sub></sub>	Q <sub>CO<sub>2</sub></sub> - $\frac{2}{3}$ Q <sub>O<sub>2</sub></sub>	$\frac{Q_{CO_2}}{Q_{CO_2}}$	$\frac{N_2}{Q_{CO_2} - Q_{CO_2} + Q_{O_2}}$	$\frac{N_2 - Q_{CO_2} + Q_{O_2}}{Q_{O_2}}$
25.....	Soybean	2% glucose	$\left\{ \begin{array}{l} N_2, 97\% O_2, 92.6\% N_2 \\ \text{Air} \\ 50\% O_2, 50\% N_2 \\ O_2 \end{array} \right\}$	0.77 1.41 1.84 2.34 3.35	0.93 1.49 2.34 3.16	..... 1.52 1.23 1.00 1.06	0.77 0.48 0.35 0.00 0.19	0.77 0.79 0.85 0.76 1.24	1.00 1.82 2.38 3.02 4.33	0.00 0.29 0.42 0.77 0.58	..... 0.94 0.85 0.99 0.55
26.....	Soybean	2% glucose	$\left\{ \begin{array}{l} N_2 \\ 50\% O_2, 50\% N_2 \\ O_2 \end{array} \right\}$	0.76 2.60 3.24	..... 2.17 2.94	..... 1.20 1.10	0.76 0.43 0.30	0.76 1.15 1.26	1.00 3.42 4.26	0.00 0.33 0.46	..... 0.46 0.47
27.....	Common vetch	2% glucose	$\left\{ \begin{array}{l} N_2, 97\% O_2, 92.6\% N_2 \\ \text{Air} \\ 50\% O_2, 50\% N_2 \\ 86\% O_2, 20\% N_2 \\ O_2 \end{array} \right\}$	0.90 2.20 3.40 5.78 7.82 8.01	..... 1.69 3.45 5.73 7.60 7.82	..... 1.30 0.98 1.01 1.03 1.02	0.90 0.51 -0.05 0.05 0.22 0.19	0.90 1.07 1.10 1.06 2.75 2.80	1.00 2.44 3.77 6.40 8.66 8.87	0.00 0.39 0.95 0.85 0.68 0.71	..... 0.69 0.83 0.45 0.27 0.27
27.....	Common vetch	Nothing	$\left\{ \begin{array}{l} \text{Air} \\ 85\% O_2, 20\% N_2 \end{array} \right\}$	2.78 6.13	2.80 6.31	1.00 0.97	-0.02 -0.18	0.91 1.92	.....	.....	.....
16.....	Crown vetch	Nothing	$\left\{ \begin{array}{l} \text{Air} \\ O_2 \end{array} \right\}$	2.77 5.47	2.41 5.06	1.15 1.08	0.36 0.41	1.16 2.10	.....	.....	.....

TABLE 8—Continued

1	2	3	4	5	6	7	8	9	10	11	12
EXP. NO.	PLANT	SUBSTRATE ADDED	GAS USED	CO <sub>2</sub> EVOLVED	O <sub>2</sub> CON- SUMED	RE- SPIR- ATORY QUO- TIENT	ALCOHOL ACCUMU- LATED	CARBOHY- DRATE CONSUMED	RATIO OF CO <sub>2</sub> EVOLVED TO CO <sub>2</sub> EVOLVED ANAERO- BICALLY	DECREASE DUE TO OXYGEN, IN ALCOHOL ACCUMULATED	DECREASE PER UNIT OF SUB- STRATE OXIDIZED IN ALCOHOL ACCUMULATED
				QCO <sub>2</sub>	QO <sub>2</sub>	$\frac{QCO_2}{QO_2}$	QCO <sub>2</sub> - QO <sub>2</sub>	QCO <sub>2</sub> - $\frac{1}{3}$ QO <sub>2</sub>	$\frac{QCO_2}{QCO_2 - \frac{N_2}{QCO_2}}$	$\frac{N_2}{QCO_2} - QCO_2 + QO_2$	$\frac{N_2 - QCO_2 + QO_2}{3QO_2}$
30.....	Korean les- pedeza	Nothing	{Air 80% O <sub>2</sub> , 20% N <sub>2</sub> O <sub>2</sub>	1.75 4.35 4.74	1.58 4.48 5.01	1.11 0.97 0.94	0.17 -0.13 -0.27	0.70 1.36 1.40	..... ..... .....	..... ..... .....	..... ..... .....
30.....	Korean les- pedeza	1% glucose	{Air 80% O <sub>2</sub> , 20% N <sub>2</sub> O <sub>2</sub>	2.41 5.28 5.51	1.72 5.13 5.45	1.40 1.03 1.01	0.69 0.15 0.06	1.26 1.86 1.88	..... ..... .....	..... ..... .....	..... ..... .....
30.....	Korean les- pedeza	5% glucose	{Air 80% O <sub>2</sub> , 20% N <sub>2</sub> O <sub>2</sub>	3.32 4.68 5.38	0.43 4.36 5.04	7.68 1.07 1.07	2.89 0.32 0.34	3.03 1.77 2.02	..... ..... .....	..... ..... .....	..... ..... .....
31.....	Korean les- pedeza	Nothing	{Air 80% O <sub>2</sub> , 20% N <sub>2</sub> O <sub>2</sub>	1.88 4.20	1.50 4.16	1.26 1.01	0.38 0.04	0.88 1.43	..... .....	..... .....	..... .....
31.....	Korean les- pedeza	0.5% alcohol	{Air 80% O <sub>2</sub> , 20% N <sub>2</sub> O <sub>2</sub>	1.48 3.33	1.40 4.17	1.05 0.80	0.08 -0.84	0.55 0.55	..... .....	..... .....	..... .....
31.....	Korean les- pedeza	2.0% alcohol	{Air 80% O <sub>2</sub> , 20% N <sub>2</sub> O <sub>2</sub>	1.63 2.77	1.66 4.06	0.98 0.68	-0.03 -1.29	0.52 0.06	..... .....	..... .....	..... .....

TABLE 9

EFFECT OF OXYGEN CONCENTRATION ON RESPIRATION OF LEGUME AND OF NON-LEGUME ROOTS

1	2	3	4	5	6	7	8	9	10	11	12
EXP. NO.	PLANT	SUBSTRATE ADDED	GAS USED	CO <sub>2</sub> EVOLVED	O <sub>2</sub> CON- SUMED	RE- SPIR- ATORY QUO- TIENT	ALCOHOL ACCUMU- LATED	CARBOHY- DRATE CONSUMED	RATIO OF CO <sub>2</sub> EVOLVED TO CO <sub>2</sub> EVOLVED ANAERO- BICALLY	DECREASE DUE TO OXYGEN, IN ALCOHOL ACCUMULATED	DECREASE PER UNIT OF SUB- STRATE OXIDIZED IN ALCOHOL ACCUMULATED
				QCO <sub>2</sub>	QO <sub>2</sub>	$\frac{QCO_2}{QO_2}$	QCO <sub>2</sub> - QO <sub>2</sub>	QCO <sub>2</sub> - $\frac{1}{3}$ QO <sub>2</sub>	$\frac{QCO_2}{QCO_2}$ $\frac{N_2}{QCO_2}$	$\frac{N_2}{QCO_2} - QCO_2 + QO_2$ $\frac{1}{3} QO_2$	
32.....	Korean les- pedeza	Nothing	$\begin{Bmatrix} N_2 \\ Air \end{Bmatrix}$ {80% O <sub>2</sub> , 20% N <sub>2</sub> }	0.48 1.20 1.40	..... 1.50 2.06	..... 0.80 0.68	0.48 -0.30 -0.66	0.48 0.20 0.03	1.00 2.52 2.94	0.00 0.78 1.14	..... 1.56 1.66
32.....	Korean les- pedeza	1% glucose	$\begin{Bmatrix} N_2 \\ Air \end{Bmatrix}$ {80% O <sub>2</sub> , 20% N <sub>2</sub> }	0.81 2.29 2.81	..... 2.35 3.01	..... 0.97 0.94	0.81 -0.06 -0.20	0.81 0.72 0.80	1.00 2.83 3.47	0.00 0.87 1.01	..... 1.11 1.01
32.....	Korean les- pedeza	2% alcohol	$\begin{Bmatrix} N_2 \\ Air \end{Bmatrix}$ {80% O <sub>2</sub> , 20% N <sub>2</sub> }	0.60 1.41 1.20	..... 1.93 2.09	..... 0.73 0.58	0.60 -0.52 -0.89	0.60 0.12 -0.19	1.00 2.33 1.99	0.00 1.12 1.49	..... 1.74 2.14
33.....	Korean les- pedeza	Nothing	$\begin{Bmatrix} Air \\ 80\% O_2, 20\% N_2 \end{Bmatrix}$	1.39 1.27	1.64 1.60	0.85 0.79	-0.25 -0.33	0.30 0.20	.....	.....	.....
33.....	Korean les- pedeza	0.5% alcohol	$\begin{Bmatrix} Air \\ 80\% O_2, 20\% N_2 \end{Bmatrix}$	1.16 1.22	1.74 1.89	0.66 0.65	-0.58 -0.67	0.00 -0.04	.....	.....	.....
33.....	Korean les- pedeza	2.0% alcohol	$\begin{Bmatrix} Air \\ 80\% O_2, 20\% N_2 \end{Bmatrix}$	1.04 1.33	1.59 1.92	0.65 0.69	-0.55 -0.59	-0.02 0.05	.....	.....	.....



concentrations of oxygen. This general result could be produced either by the complete oxidation of a greater proportion of the substrate consumed, by the consumption of more substrate, or by both. The R.Q.'s given in the two tables indicate that the result was at least partly due—up to a certain point—to more complete oxidation and after that to increased consumption of substrate, for in the case of nodules supplied with glucose the R.Q.'s decreased with increased oxygen concentration until a value of approximately 1.00 was reached, and then remained constant. With roots the data are less complete and less conclusive, but indicate a similar behavior, with a somewhat lower minimum value; in particular, where alcohol was added this minimum appears to have been about 0.67.

The fact that  $\text{CO}_2$  evolution and  $\text{O}_2$  consumption increased in the upper range of  $\text{O}_2$  concentrations, while the R.Q. remained constant or decreased, shows that in this range substrate consumption was increased by increases in concentration of oxygen. The question as to whether or not additional  $\text{O}_2$  increased consumption of substrate in the low range of oxygen concentrations, where more complete oxidation is also a factor, is answered for nodules with added glucose in columns 8 and 9 of table 8. It is shown here that, although alcohol accumulation decreased with increased  $\text{O}_2$  concentration, carbohydrate consumption increased throughout the entire range of oxygen concentrations. For nodules with added alcohol, however, and for roots—both with and without added alcohol (table 9)—the situation with regard to carbohydrate consumption was reversed; that is, less carbohydrate was consumed at high oxygen concentrations than at low ones, although the alcohol accumulation continued to decline. In fact the alcohol accumulation values became negative, indicating a consumption of alcohol. While the data are hardly sufficient to be positive, it seems very likely that here also the increase in  $\text{O}_2$  concentration led to an increase in total substrate consumed, but with alcohol as the main substrate.

The observation just given concerning the effect of increased oxygen concentration on the consumption of carbohydrate is verified by the figures in column 10 of both tables, where the amount of  $\text{CO}_2$  evolved at different oxygen concentrations is compared with that evolved anaerobically in those experiments which included anaerobic cultures. With either nodules or roots with glucose added, in all concentrations of oxygen producing complete aerobiosis (where the R.Q. equaled approximately 1.00) the ratio of  $\text{CO}_2$  evolved aerobically to that evolved anaerobically was 3.00 or greater. This means that if the substrate being utilized was glucose, as is usually considered to be the case in plant respiration, as much or more glucose was consumed in the presence of oxygen as in its absence, since only three times as much  $\text{CO}_2$  is produced by the complete oxidation of carbohydrate as by its fermentation to alcohol. The size of this quotient is of less significance where alcohol was added, of course, since in that case the original substrate appears to have been chiefly this compound.

This relationship can be analyzed further by the functions given in columns 11 and 12. Column 11 gives the decrease, due to oxygen, in alcohol accumulated, and column 12 gives the amount of this decrease per unit of substrate oxidized. This latter function, sometimes known as the Meyerhof oxidation quotient, equals 1.00 if the effect of the oxygen is merely to oxidize alcohol (or similar intermediate); equals less than 1.00 if in addition carbohydrate consumption is increased; and equals more than 1.00 if carbohydrate consumption is decreased or alcohol disappears more rapidly than it is oxidized to  $\text{CO}_2$  and  $\text{H}_2\text{O}$  (as, for example, by a coupled oxidative resynthesis to carbohydrate such as has been observed occasionally).

In all cases, either with nodules or roots, where glucose was added the decrease in alcohol accumulated was 1.00 or less for each unit of substrate oxidized. The evidence therefore indicates that under these circumstances oxygen stimulated the consumption of glucose. On the other hand, in the one instance with roots respiring only their own food reserves and in the one where alcohol was added, the decrease in alcohol accumulated per unit of substrate oxidized was definitely greater than 1.00. From a consideration of the respiratory quotients, mentioned earlier, it seems likely that the result is due to a decrease in carbohydrate consumption rather than to a resynthesis of carbohydrate from alcohol. Unfortunately no data are at hand which would permit the calculation of this function for nodules cultured in alcohol, but there is no reason to suspect that they would be contradictory.

Some of these conclusions are naturally only tentative, because of the limited amount of work on which they are based.

### Discussion

#### SIMILARITY BETWEEN NODULES AND SMALL ROOTS IN FORMATION AND UTILIZATION OF ALCOHOL

The well-known difference in the nitrogen metabolism of roots and of nodules with their inclosed bacteria shows that some sort of fundamental difference exists between the nitrogen-fixing tissue and the ordinary root tissue. The results reported here, in agreement with the data given in the second paper (2) of this series, indicate strongly that so far as respiration is concerned no such differences exist. It is true that the respiratory quotient is lower in roots and that a sparing action of oxygen on carbohydrate is evident, as opposed to the opposite effect in nodules, but the experiments indicate that the only thing necessary to bring about the difference is a difference in the amounts of carbohydrate and alcohol (intermediate) present in the tissues. The differences appear to be those of degree only, and not of kind, with the respiration process fundamentally the same in the two tissues. The roots respire like nodules supplied with alcohol, although in view of data already presented (2) the question is still open as to whether the results are

due to an abundance of alcohol or to a lack of carbohydrate, with accompanying oxidation of protein. The oxidation of protein, however, would imply that the roots were oxidizing themselves away, a thing that seems improbable in growing organs. On the other hand, the oxidation of alcohol or other intermediate would raise the question as to where it comes from, since it is generally understood that carbonaceous foods are transported from the green tissues as sugar. But when it is considered that only fine roots were used, this question loses much of its importance, since the substrate had to traverse the thick roots in which, as shown by one or two experiments not otherwise mentioned in this paper, the conditions were at least partially anaerobic. Consequently it does not seem unreasonable to suppose that some of the substrate may have undergone fermentation during its passage, so that much of that which reached the fine roots was in the form of alcohol.

#### ROLE OF ALCOHOL IN RESPIRATION

It has long been recognized that in the absence of oxygen, alcohol and  $\text{CO}_2$  are the chief products of the respiration of sugars by most higher plant tissues, and that in its presence the corresponding products are  $\text{CO}_2$  and water. The related point—as to whether or not alcohol is an intermediate product in normal aerobic respiration—has often been discussed but cannot yet be said to be settled. In connection with this point, the oxidizability of alcohol in the tissues is of fundamental importance, for obviously the compound can scarcely function as an intermediate if it cannot be oxidized. Early work on this phase of the question was done by MAZÉ and PERRIER (14), KOSTYTSCHEW (9, 10), and ZALESKI (21), all of whom gave evidence that alcohol is oxidizable in the tissues of higher plants. TAKAHASHI (16), however, had previously implied by analogy with animal tissues (but without experimental proof) that it is more slowly oxidized than sugar, and KOSTYTSCHEW's results included some cases in which no oxidation occurred. He concluded that alcohol is oxidized slowly and incompletely; and his later attitude (11) seems to have been dominated even more by this idea, since he said that he "has shown that ethyl alcohol is oxidized by seed plants with difficulty or not at all." Moreover, the same idea seems to prevail in much of the contemporary work on the subject (3, 11, 15, 17).

In the last decade, however, scattered data have appeared which indicate that the early results were correct. BUGAJEWSKY (4) showed that alcohol accumulates in germinating pea seeds in the absence of oxygen and disappears in the presence of the latter, and CHATTERJI (6) likewise showed that the  $\text{CO}_2$  output of certain leaves is increased by infiltrating them with dilute solutions of alcohol. While these results do not prove that oxidation of alcohol took place in the plant tissues, they do point that way. WETZEL (20) and THUNBERG (18) have investigated the enzyme system involved. THUNBERG showed that the seeds of many plants con-

tain an alcohol dehydrogenase, and WETZEL claimed to have found in some plants a complete enzyme system for the complete oxidation of alcohol through acetaldehyde and acetic acid. (Unfortunately the experimental basis for the claim is not given in the only paper (20) discussing it that the writers have seen.)

The results obtained in the present study definitely favor the idea that alcohol is oxidizable in the tissues of higher plants. It was found to accumulate in the tissues under anaerobic conditions and to disappear under aerobic conditions, and the R.Q. of tissues supplied alcohol artificially was such as would be expected if the alcohol which disappeared had been oxidized to  $\text{CO}_2$  and  $\text{H}_2\text{O}$ . Moreover, the ratio of aerobically produced to anaerobically produced  $\text{CO}_2$ , and the decrease—caused by oxygen—in alcohol accumulated per unit of carbohydrate oxidized when an abundance of carbohydrate was present, indicate that no more alcohol disappeared under the influence of oxygen than was oxidized to  $\text{H}_2\text{O}$  and  $\text{CO}_2$ . The most reasonable explanation for the disappearance of the alcohol therefore seems to be not that it was resynthesized to a fermentable substrate but that it was oxidized to  $\text{CO}_2$  and water. The evidence therefore indicates that, whether or not alcohol normally occurs as an intermediate compound in the aerobic respiration of carbohydrate, it can be oxidized completely by many higher plant tissues.

The results also indicate that oxygen may exert a sparing effect or no sparing effect on the carbohydrate respired in one and the same tissue, apparently depending on the relative abundance of carbohydrate and intermediate present in the tissue. Since the R.Q.'s suggest that the sparing effect when present was not due to any oxidative reversion of intermediates, the suggestion remains that in tissues in which much intermediate has accumulated the  $\text{O}_2$  in some way reduces the primary breakdown of the carbohydrate. This concept is supported by the fact that alcohol and sugar appear to be competitively oxidized in these tissues, with a probable preferential oxidation of alcohol.

It is not entirely certain that the sparing effect<sup>4</sup> discussed here is identical with the one sometimes reported in plant tissues, but there is apparently no special reason for thinking that it is not; and if it should be found to be the same it would explain in many cases, without assuming any fundamental difference in the mechanism of respiration, why the sparing effect sometimes occurs and sometimes does not. In that case it will be found if for some reason intermediates of fermentative origin have accumulated sufficiently and will be absent if intermediates are absent, or are present in small amounts only. MARSH and GODDARD (12) have recently reported in carrot tissue a sparing effect of oxygen, accompanied by

<sup>4</sup> Some of the phenomena under discussion here bear resemblance to the frequently discussed Pasteur reaction. However, a considerable amount of uncertainty seems to exist concerning the exact definition of the Pasteur reaction and of some of the terms commonly used in discussing it (5, 8, 19). For this reason it has seemed best to discuss the significance of the data without reference to this reaction as such and so far as possible with descriptive terms instead of with the less definite conventional ones.

R.Q. values at 5 per cent  $O_2$  or above, of usually less than 0.90. Apparently the sparing effect in this species also may be dependent upon the accumulation of intermediates in the tissue.

### Summary

A series of experiments by both manometric and analytical methods has been conducted on the production and oxidation of ethyl alcohol by legume nodules and the data compared with similar data from small roots of legumes and of non-legumes. The following results were obtained:

1. Ethyl alcohol was present in small amounts in all tissues studied. Under anaerobic conditions it accumulated and  $CO_2$  was evolved in approximately the proportions which would result from an alcoholic fermentation of sugar.
2. Under aerobic conditions, with alcohol added to the medium, part of the alcohol disappeared in a few hours and the R.Q. of the tissues was reduced by an amount depending on the concentration of the alcohol. At the higher concentrations it was commonly reduced nearly to that R.Q. (0.67) corresponding to the complete oxidation of alcohol. These results indicate that the alcohol which disappeared was oxidized completely.
3. Added alcohol increased oxygen consumption of both nodules and roots but not nearly enough to account for the change in R.Q. It is suggested that the alcohol consumption replaced and reduced the consumption of the other substrate present.
4. When glucose and alcohol were made available together in the medium, each affected the R.Q. in the same way as it did in the absence of the other, again without affecting the oxygen consumption proportionately, thus indicating that each inhibited competitively the oxidation of the other.
5. For nodules, either without added substrate or with glucose added, and for small roots with glucose added, the absence of a sparing action of oxygen on carbohydrate (and consequently of any oxidative reconversion of the products of fermentation to carbohydrate or similar compound) was indicated (*a*) by increased  $CO_2$  evolution and  $O_2$  consumption due to increasing concentrations of oxygen; (*b*) by similarly induced increases in carbohydrate consumption; (*c*) by a ratio equal to or greater than 3.00 between the  $CO_2$  produced under complete aerobiosis and that produced anaerobically; and (*d*) by the failure of the decreases, due to oxygen, in alcohol accumulation to exceed the carbohydrate oxidized.
6. On the contrary, for nodules with sufficient added alcohol and for small roots without added substrate or with alcohol added, the presence of a sparing action of oxygen on carbohydrate (in spite of increased total substrate consumption) was indicated by a decrease in carbohydrate consumption with increasing oxygen concentration and by a decrease in alcohol accumulation which was greater than the

carbohydrate oxidized. Since the R.Q.'s indicate the absence of any oxidative reconversion of alcohol, it is suggested that the oxygen may have reduced in some way the primary breakdown of the carbohydrate.

7. The differences between the behavior of nodules and of roots in respiration are not thought to be fundamental. The roots behaved much like nodules with added alcohol.

8. The definite demonstration that alcohol is oxidized by the tissues of higher plants removes an important objection to considering it as an intermediate in normal plant respiration, but of course does not prove that it is an intermediate.

9. It is suggested that the occasionally observed sparing action of oxygen on carbohydrate in respiring plant tissues may be dependent on a high concentration of intermediate. If so, no fundamental difference in the mechanism of respiration need be assumed between that in tissues where the sparing action occurs and that in those from which it is absent.

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# ANATOMICAL AND PHYSIOLOGICAL RESPONSES OF THE TOMATO TO VARYING CONCENTRATIONS OF SODIUM CHLORIDE SODIUM SULPHATE, AND NUTRIENT SOLUTIONS

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(WITH TEN FIGURES)

## Introduction

Plants grown in soil solutions containing high concentrations of soluble salts commonly exhibit markedly unfavorable growth responses. These may be caused by (a) the toxic effect of high concentration of a single ion, (b) the interaction of two or more ions present in high concentrations, or (c) the combined effect of high total salt concentration.

Experiments were set up to determine (a) the character of the anatomical and physiological responses of tomato plants when grown in a series of nutrient solutions containing progressively higher concentrations of the major constituent salts, and (b) the responses these plants exhibit when grown in sodium chloride and sodium sulphate solutions having osmotic concentrations equal to those of the solutions in the nutrient series.

## Material and methods

The tomato plant was selected because it can be easily grown under greenhouse conditions and has been used extensively in studies of nutrition. The plants were grown in sand cultures in pots devised by EATON (2). These are provided with a reservoir holding approximately 13 liters of solution, which can be circulated at regular intervals by means of compressed air passing through an ejector. A gage on the reservoir indicates the level of the solution, and water was added daily to compensate for losses resulting from transpiration and evaporation. A time clock controls flushing of the cultures, and was set to circulate the liquid at 2-hour intervals from 6:00 A.M. to 6:00 P.M., with one irrigation at midnight. A flow for 3-5 minutes more than displaces the solution held in the sand.

## NUTRIENT AND SALT SOLUTIONS

EXPERIMENT I.—A four-salt nutrient solution was used containing  $\text{Ca}(\text{NO}_3)_2$ ,  $\text{KNO}_3$ ,  $\text{MgSO}_4$ , and  $\text{KH}_2\text{PO}_4$ , and the total salt concentration was adjusted to 0.5 atmosphere osmotic concentration (table 1). This solution was used as the base

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nutrient in the controls and as the initial solution for all cultures until the seeds had germinated. It also constituted the nutrient portion of the sodium chloride and sodium sulphate solutions.

Three series of solutions were used:

1. Base nutrient series: Four solutions in which the nutrient salts were increased at each level to produce osmotic concentrations of 0.5, 1.5, 3, and 4.5 atmospheres.
2. Sodium chloride series: Three solutions consisting of the base nutrient at 0.5 atmosphere plus NaCl sufficient to result in osmotic concentrations of 1.5, 3, and 4.5 atmospheres.
3. Sodium sulphate series: Three solutions consisting of the base nutrient at 0.5 atmosphere plus  $\text{Na}_2\text{SO}_4$  sufficient to result in osmotic concentrations of 1.5, 3, and 4.5 atmospheres.

TABLE 1  
COMPOSITION OF SOLUTIONS USED IN EXPERIMENT I

CONCENTRATION OF SOLUTIONS (ATMOSPHERES)	BASE NUTRIENT SERIES CONSISTING OF FOLLOWING SALTS (MILLIMOLES PER LITER)				CONCENTRATION OF SOLUTIONS (ATMOSPHERES)	MILLIMOLES ADDED TO BASE NUTRIENT SOLUTION AT 0.5 ATMOSPHERE CONCENTRATION	
	$\text{Ca}(\text{NO}_3)_2$	$\text{KNO}_3$	$\text{MgSO}_4$	$\text{KH}_2\text{PO}_4$		SODIUM CHLORIDE SERIES NaCl	SODIUM SULPHATE SERIES $\text{Na}_2\text{SO}_4$
0.5.....	2.6	5.2	2.6	0.65	1.5.....	20.0	15.0
1.5.....	7.2	14.4	7.2	1.8	3.0.....	52.0	40.0
3.0.....	15.8	31.6	15.8	3.95	4.5.....	89.0	71.0
4.5.....	24.8	49.6	24.8	6.2			

The proportions of the constituent salts used in the various solutions are expressed in millimoles per liter. In determining the amounts of salts needed to produce 0.5 atmosphere osmotic concentration, c.p. salts and distilled water were used. When commercial salts and Riverside tap water were used, the proportions produce an osmotic concentration of approximately 0.6 atmosphere. Minor elements were added in the following amounts: boron 1.0 p.p.m., zinc 0.05 p.p.m., manganese 0.5 p.p.m., molybdenum 0.05 p.p.m., and copper 0.02 p.p.m. Iron as citrate was supplied as needed in 1 p.p.m. additions.

EXPERIMENT II.—The solutions used were essentially like those in experiment I, but the quantities of salts needed to produce 0.5 atmosphere osmotic concentration were determined on the basis of commercial salts and tap water. The amount of  $\text{KH}_2\text{PO}_4$  was kept at a constant level (1.8 mm./l.) in all solutions, to avoid the heavy precipitation that occurred in the higher base nutrient cultures in experiment I. In order to explore further the effect of high salt concentrations, an additional culture with salts adjusted to 6 atmospheres osmotic concentration was

added to each series. The base nutrient series was set up at five levels: 0.5, 1.5, 3, 4.5, and 6 atmospheres osmotic concentration; and the sodium chloride and sodium sulphate series at four levels: 1.5, 3, 4.5, and 6 atmospheres. The composition of the solutions is indicated in table 2. Minor elements and iron were added as in experiment I, and additional increments of manganese (0.5 and 1 p.p.m.) were supplied during the latter part of the run, when symptoms of manganese deficiency appeared in the leaves of the larger plants.

TABLE 2  
COMPOSITION OF SOLUTIONS USED IN EXPERIMENT II

CONCENTRATION OF SOLUTIONS (ATMOSPHERES)	BASE NUTRIENT SERIES CONSISTING OF FOLLOWING SALTS (MILLIMOLES PER LITER)				CONCENTRATION OF SOLUTIONS (ATMOSPHERES)	MILLIMOLES ADDED TO BASE NUTRIENT SOLUTION AT 0.5 ATMOSPHERE CONCENTRATION	
	Ca(NO <sub>3</sub> ) <sub>2</sub>	KNO <sub>3</sub>	MgSO <sub>4</sub>	KH <sub>2</sub> PO <sub>4</sub>		SODIUM CHLORIDE SERIES NaCl	SODIUM SULPHATE SERIES Na <sub>2</sub> SO <sub>4</sub>
0.5.....	1.3	2.6	1.3	1.8	1.5.....	20.0	15.0
1.5.....	7.3	14.6	7.3	1.8	3.0.....	54.0	40.0
3.0.....	16.1	32.2	16.1	1.8	4.5.....	90.0	71.0
4.5.....	26.0	52.0	26.0	1.8	6.0.....	120.0	100.0
6.0.....	35.1	70.2	35.1	1.8			

#### REPLACEMENT AND MAINTENANCE OF SOLUTIONS

To avoid seedling injury, at the initiation of each experiment all pots were supplied with the base nutrient at the 0.5 atmosphere level, and the higher osmotic concentrations were obtained by a series of increases extending over a 10-day period. Complete changes of solutions were made as follows: experiment I—October 5 and 23, and November 7; experiment II—November 21, December 7 and 26, and January 7. At intervals between solution changes the osmotic concentration of each solution was determined by the freezing-point depression method.

The H-ion concentration was determined with a Beckman pH meter on alternate days, or daily when the plants were larger. Solutions were adjusted to pH 7.0 by additions of HNO<sub>3</sub> or NaOH when there was a variation of more than 0.5 pH. In experiment II, KOH was used instead of NaOH. Until the plants were large, it was never necessary to add more than 5 cc. of HNO<sub>3</sub>, and 10 cc. was the greatest amount supplied at one time.

#### ENVIRONMENTAL FACTORS (EXPERIMENTS I AND II)

The cultures were grown under greenhouse conditions, and records of temperature and relative humidity were obtained with a hygrothermograph. During the

course of experiment I (September 23 to November 11, 1939) the mean maximum temperature was 88.6° and the mean minimum 61.6° F. Up to the time of the first harvest in experiment II (November 15, 1939 to January 2, 1940) the mean maximum and minimum temperatures were only slightly less than those of experiment I, 86.5° and 59.4° F., respectively. The final 4 weeks of the second experiment (January 2 to 30, 1940) were cooler, 78° and 58.1° F. The mean values for relative humidity were: experiment I—maximum 70.9 per cent, minimum 28.7 per cent; experiment II—77.8 per cent and 39.6 per cent up to the first harvest and 81.3 per cent and 56.1 per cent for the final 4 weeks.

The major climatic variation between the two experiments was the amount of solar and sky radiation. At Riverside, California, the mean total daily radiation for the period of the first experiment was 354.8 gm. calories per square centimeter of horizontal surface, while for the equivalent period of the second experiment it was 256 gm. calories. There was a further reduction in radiation during the final 4 weeks of the second experiment when the daily mean was 221 gm. calories.<sup>3</sup> These values were taken outdoors, and somewhat lower ones would obtain in the greenhouse, where the experiments were conducted.

#### PLANTING DATA (EXPERIMENTS I AND II)

A commercial strain of the Marglobe variety of the tomato was used, and all trials were run in duplicate.

The seeds of experiment I were planted September 23, 1939, and the cultures given identical amounts of water and nutrient solution until September 30. At this time all except the control pots were increased to 1.5 atmosphere osmotic concentration. On October 3, all but the controls and 1.5 atmosphere cultures were adjusted to 3 atmosphere concentration; and on October 5, the 4.5 atmosphere cultures were brought to full strength. In this manner the three series were established as follows: the base nutrient series at 0.5, 1.5, 3, and 4.5 atmospheres osmotic concentration and the sodium chloride and sodium sulphate series at 1.5, 3, and 4.5 atmospheres.

The seeds of experiment II were planted November 15, and on November 25 all except the control pots were adjusted to 1.5 atmosphere concentration. Increases in the concentration of the cultures were made as in experiment I. The 3 atmosphere level was reached November 27, the 4.5 on November 29, and the 6 on December 1. The final adjustment established the three series at the following levels: the base nutrient at 0.5, 1.5, 3, 4.5, and 6 atmospheres osmotic concentration and the sodium chloride and sodium sulphate series at 1.5, 3, 4.5, and 6 atmospheres.

<sup>3</sup> These figures supplied through the courtesy of Dr. E. R. PARKER, at Citrus Experiment Station, Riverside, where a pyroheliometer is maintained.

## Experimentation

## SEEDLING RESPONSES

Approximately twenty seedlings were started in each culture. These were thinned selectively to provide three uniform plants. Although the seedlings removed were the least uniform in each culture, their average heights and total fresh and dry weights did provide an index of the early responses to the nutrient and salt solutions used in the three series.

TABLE 3  
EXPERIMENT II: SEEDLING HARVEST DATA. FRESH AND DRY WEIGHTS  
AND PERCENTAGE OF DRY MATTER

SERIES AND CONCENTRATION OF SOLUTIONS (ATMOSPHERES)		HARVEST DECEMBER 7, 1939			HARVEST DECEMBER 13, 1939		
		FRESH WT. 12 PLANTS (GM.)	DRY WEIGHT (GM.)	DRY MATTER (PER CENT)	FRESH WT. 6 PLANTS (GM.)	DRY WEIGHT (GM.)	DRY MATTER (PER CENT)
Base nutrient	{ 0.5.....	11.53	0.84	7.3	22.95	1.81	7.9
	{ 1.5.....	13.66	1.11	8.1	27.00	1.99	7.4
	{ 3.0.....	9.05	0.76	8.5	19.31	1.60	8.3
	{ 4.5.....	5.25	0.47	9.0	6.61	0.64	9.7
	{ 6.0.....	5.14	0.51	10.0	6.72	0.72	10.8
NaCl	{ 1.5.....	9.84	0.70	7.2	17.53	1.33	7.6
	{ 3.0.....	7.05	0.45	6.4	13.24	0.68	5.2
	{ 4.5.....	7.85	0.55	7.1	12.24	0.72	5.9
	{ 6.0.....	6.75	0.41	6.1	8.37	0.63	7.6
Na <sub>2</sub> SO <sub>4</sub>	{ 1.5.....	8.43	0.67	8.0	20.28	1.54	7.6
	{ 3.0.....	7.72	0.60	7.9	12.17	0.77	6.4
	{ 4.5.....	5.80	0.46	8.0	6.24	0.54	8.8
	{ 6.0.....	3.59	0.31	8.9	3.15	0.28	9.1

In both experiments there was a progressive growth depression with increasing concentration in the sodium chloride and sodium sulphate series. In the base nutrient series the seedlings grown in the 1.5 atmosphere solution were larger and heavier than those in the 0.5 atmosphere culture. In both seedling harvests of experiment II the lower percentages of dry weight in the cultures of the sodium chloride series, as compared with those of equivalent osmotic concentrations in the other two series, indicated a greater degree of succulence in the high chloride solutions (table 3).

## PHYSIOLOGICAL AND CHEMICAL DATA

EXPERIMENT I.—Three plants in each culture were maintained until November 13, a growing period of 51 days.

Except for the 1.5 atmosphere culture in the base nutrient series, in which the plants were taller than those at the 0.5 level, there was a decrease in height at high

concentrations of the culture solution in all series. The difference in height between the plants in the 1.5 and 4.5 atmosphere solutions was least in the base nutrient and greatest in the sodium sulphate series.

In all series there was a significant reduction in the diameter of the stem at the high concentrations. This was least in the sodium chloride and greatest in the sodium sulphate series (table 4).

Except for the 1.5 atmosphere culture in the base nutrient series, the total fresh and dry weights of the tops decreased at high concentrations. The fact that the 1.5 atmosphere base nutrient solution promoted greater vegetative growth than that of the 0.5 atmosphere culture suggests that the supply of nutrient salts, especially nitrogen, was more nearly optimum at the 1.5 atmosphere level. All cultures,

TABLE 4  
EXPERIMENTS I AND II: AVERAGE DIAMETER OF FIRST INTERNODE OF STEM\*

CONCENTRATION OF SOLUTIONS (ATMOSPHERES)	SERIES					
	BASE NUTRIENT (MM.)		SODIUM CHLORIDE (MM.)		SODIUM SULPHATE (MM.)	
	I	II	I	II	I	II
0.5.....	10.3	8.0	.....	.....	.....	.....
1.5.....	9.9	8.2	9.7	8.2	9.0	7.5
3.0.....	8.8	7.5	9.1	6.8	7.2	6.0
4.5.....	7.2	6.7	7.5	6.5	4.5	3.2
6.0.....	.....	6.0	.....	5.4	.....	2.0

\* Average of four stems in each culture.

however, except the high concentrations in the base nutrient series, were supplied with the base nutrient solution at the 0.5 atmosphere level, and no symptoms of nitrogen deficiency were observed.

EXPERIMENT II.—Three plants per culture were maintained until January 2, when two plants from each pot were harvested. The length of the growing period, 48 days, was approximately that of experiment I. The remaining plant in each culture was harvested January 30, 75 days after planting. In all series, at the high levels of concentration the stems were shorter and smaller in diameter than at the control or 1.5 atmosphere levels (table 4).

As in experiment I, the fresh and dry weights of tops decreased with increasing concentration, except in the 1.5 atmosphere base nutrient solution (fig. 1).

In both experiments the percentage of dry matter increased with increasing concentration in the sodium sulphate and base nutrient series and remained practically unchanged in the sodium chloride series. This indicates greater succulence in the high chloride plants than in those of the base nutrient and sodium sulphate series at osmotic concentrations (fig. 2).

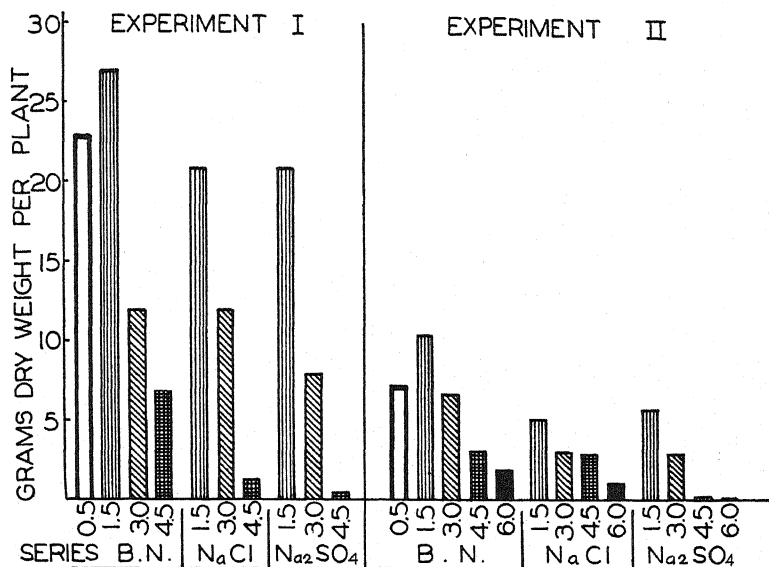


FIG. 1.—Experiments I and II. Average dry weights of tops per plant at harvests of November 10 and January 2. Figures below columns indicate atmospheres osmotic concentration of culture solutions.

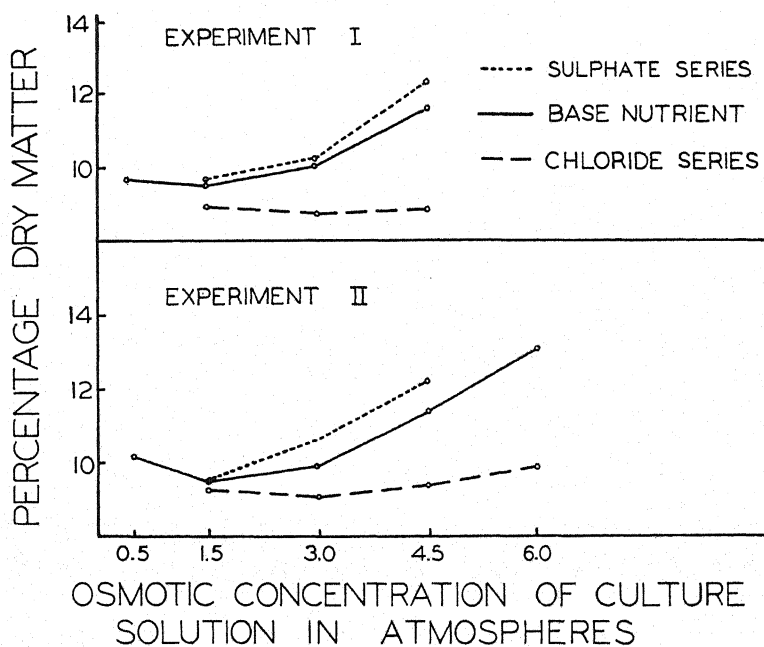


FIG. 2.—Experiments I and II. Percentage dry matter of tops at increasing levels of osmotic concentration in each series.

In corresponding cultures the plants in experiment I were shorter and stockier than those in experiment II and produced greater fresh and dry weights of tops. There was relatively little variation in temperature during the comparable periods of the two experiments, and these differences can probably be ascribed to the light factor, since the solar and sky radiation was 31.5 per cent greater in the first experiment than in the second.

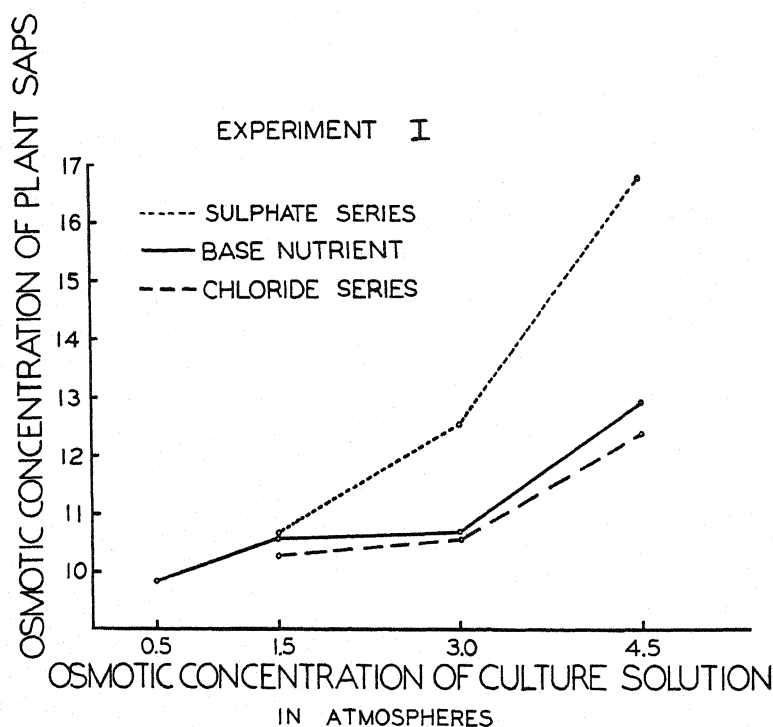


FIG. 3.—Experiment I. Relation between osmotic concentration of plant saps and that of culture solutions.

In both experiments observations on flower-bud formation indicated a definite reduction or retardation in formation in the high sodium chloride solutions and a more pronounced effect in the high sodium sulphate cultures, in which no flower buds were produced up to the time of harvest.

In experiment II all plants except those grown in the 4.5 and 6 atmosphere sodium sulphate solutions had produced evident flower buds at the time of final harvest, but bud formation was retarded at the higher levels in the base nutrient and sodium chloride series. The plants in the 6 atmosphere sodium sulphate solution died before the final harvest. Anthesis was correspondingly delayed at the higher concentrations. The number of inflorescences was greatest in the 1.5 and 3

atmosphere cultures in the base nutrient series and at the 1.5 atmosphere level in the sodium chloride and sodium sulphate series.

The osmotic concentrations of the expressed sap were determined at the conclusion of experiment I. In all series the sap concentration increased with increasing concentration of the culture solution. At isosmotic solution pressures the sap concentrations were highest in the sodium sulphate series. There was close correspondence between the base nutrient and sodium chloride series, the latter having slightly lower sap concentrations (fig. 3).

In experiment II the accumulation of Cl, S, and Na was determined for both harvests of the sodium chloride and sodium sulphate series.<sup>4</sup> Determinations were also made for the 0.5 and 1.5 atmosphere levels of the base nutrient series for purposes of comparison (table 5).

TABLE 5  
EXPERIMENT II: BASE NUTRIENT SERIES  
ACCUMULATION OF CL, S, AND NA

CONCENTRATION OF SOLUTIONS (ATMOSPHERES)	MILLIEQUIVALENTS PER KILO DRY WEIGHT OF TOPS					
	FIRST HARVEST			SECOND HARVEST		
	CL	S AS SO <sub>4</sub>	NA	CL	S AS SO <sub>4</sub>	NA
0.5.....	197	558	147	154	450	127
1.5.....	112	634	65	151	681	54

In the sodium chloride series there was increasing accumulation of Na and Cl in the tops of the plants with increasing concentration of the culture solution (table 6). In the sodium sulphate series the accumulation of Na increased progressively at the higher concentrations, but there was no marked trend with respect to S intake (table 7).

The results obtained for S accumulation in the sodium sulphate series are not in agreement with unpublished results obtained for tomatoes by EATON<sup>5</sup> at this laboratory. In out-of-doors sand cultures, he found 150, 177, 222, and 299 m.e. of SO<sub>4</sub> per liter in the sap of tomatoes grown in solutions containing 2.68, 50, 160, and 250 m.e. of SO<sub>4</sub> per liter, respectively.

In both sodium series the intake of Na on a dry-weight basis indicates a significant relation to the amount available in the culture solutions. In solutions with

<sup>4</sup> A.O.A.C. methods for Cl and S analyses.

Na analysis: BARBER, H. H., and KOLTHOFF, I. M., A specific reagent for the rapid gravimetric determination of sodium. Jour. Amer. Chem. Soc. 50:1625-1631. 1928.

<sup>5</sup> F. M. EATON, private communication.



equal concentrations of Na, the accumulation of Na was greater in the sodium sulphate cultures than in those of the corresponding sodium chloride series (fig. 4). A composite picture of the accumulation of Cl, S, and Na on the basis of dry weight of tops is shown in figure 5.

TABLE 6  
EXPERIMENT II: SODIUM CHLORIDE SERIES  
ACCUMULATION OF CL AND NA

CONCENTRATION OF SOLUTIONS (ATMOSPHERES)	MILLIEQUIVALENTS PER KILO DRY WEIGHT OF TOPS			
	FIRST HARVEST		FINAL HARVEST	
	CL	NA	CL	NA
1.5.....	880	653	754	563
3.0.....	1090	1320	911	934
4.5.....	1480	1780	1340	1445
6.0.....	1800	2285	1520	1645

TABLE 7  
EXPERIMENT II: SODIUM SULPHATE SERIES  
ACCUMULATION OF S AND NA

CONCENTRATION OF SOLUTIONS (ATMOSPHERES)	MILLIEQUIVALENTS PER KILO DRY WEIGHT OF TOPS			
	FIRST HARVEST		FINAL HARVEST	
	S AS SO <sub>4</sub>	NA	S AS SO <sub>4</sub>	NA
1.5.....	828	1090	694	709
3.0.....	749	2180	506	1585
4.5.....	1080	2565	574	2275

#### ANATOMICAL RESPONSES

At harvest all plants were sampled to obtain representative material for anatomical analysis. In selecting material, care was exercised to establish a definite reference point, so that the structures examined would be comparable. The stem segments were selected from the median portion of the basal internode immediately above the cotyledonary node. Mature leaves were selected, and a rectangular segment of the terminal leaflet, oriented so as to include a portion of the midvein, was taken at a median point in the lamina.

The material was fixed with Navashin's solution, dehydrated in an ethyl-tertiary butyl alcohol series, and infiltrated with a paraffin-beeswax-rubber mix-

ture. Immediately after placing the material in the fixative, air was evacuated from the tissues with a vacuum pump. Sections were cut at  $15\ \mu$  and stained with a modified Flemming's triple stain. The photomicrographs of all series were taken at the same magnification.

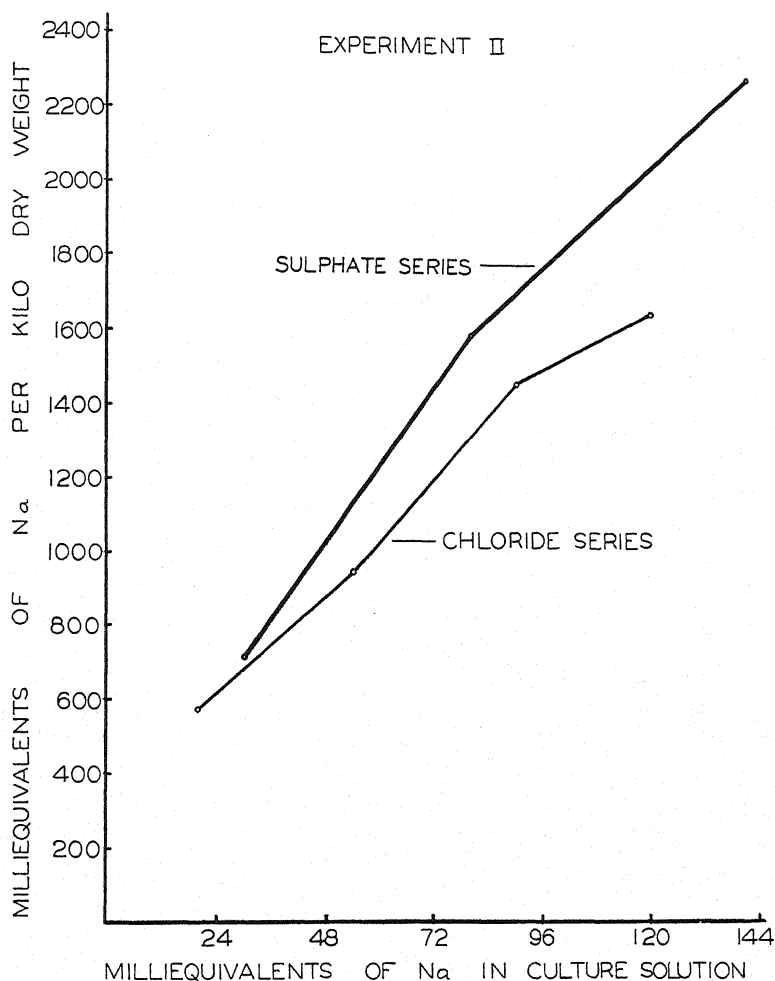


FIG. 4.—Experiment II. Sodium chloride and sodium sulphate series. Relation between concentration of sodium in culture solution and accumulation of sodium in tops.

**STEM ANALYSIS.**—The tomato stem has a dissected siphonostele consisting of three or four bicollateral bundles which at first are separated by broad medullary rays. As the stem matures the fascicular and interfascicular cambiums form a continuous cylinder, and a solid zone of secondary vascular tissue is produced. The mechanical tissues consist of a band of collenchyma lying centrad to a narrow

zone of chlorenchyma, and the outer and inner pericyclic fibers. A very small number of inner pericyclic fibers was differentiated (5).

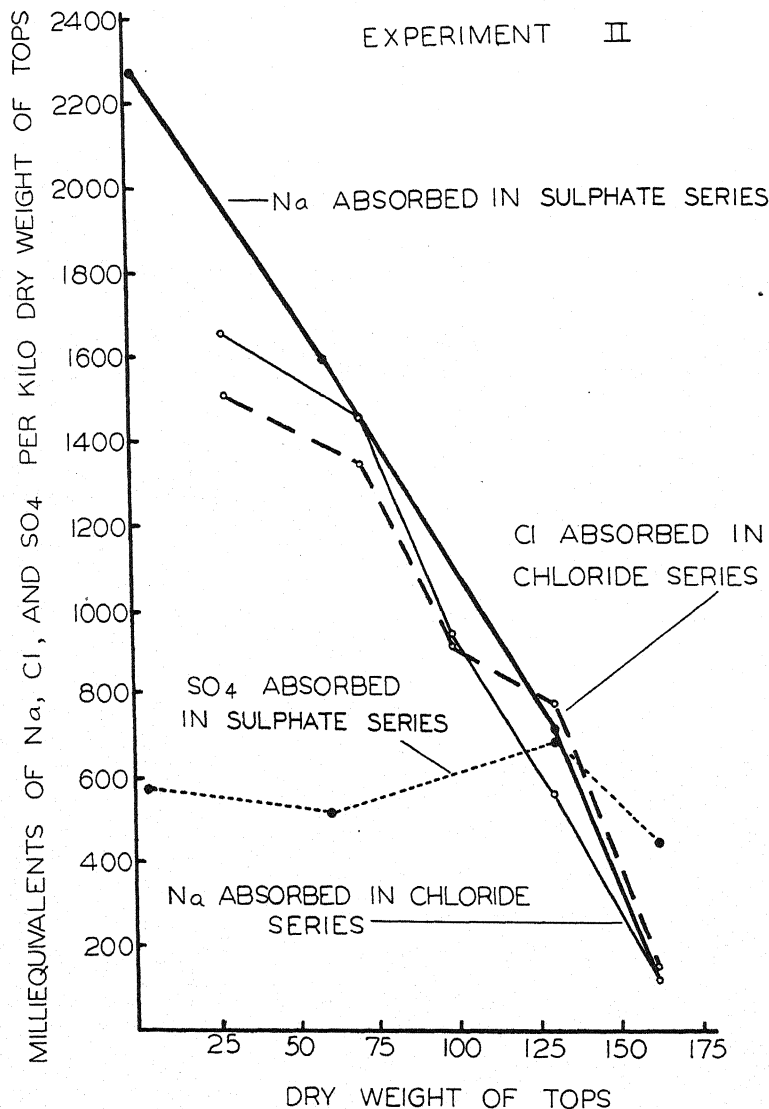


FIG. 5.—Experiment II. Sodium chloride and sodium sulphate series. Relation between accumulation of sodium, chloride, and sulphate ions and dry weight of tops.

With few exceptions the anatomical responses in corresponding cultures of the two experiments agreed with respect to their general character and differed only in degree. For this reason the results obtained in experiments I and II are dis-

cussed jointly for each series. Additional observations are included for the 6 atmosphere level in experiment II.

The smaller diameters of stems grown in solutions of high salt concentration were the result of differential reduction in their tissue systems. In general, the reduction of the vascular tissues was greater than that of the parenchymatous tis-

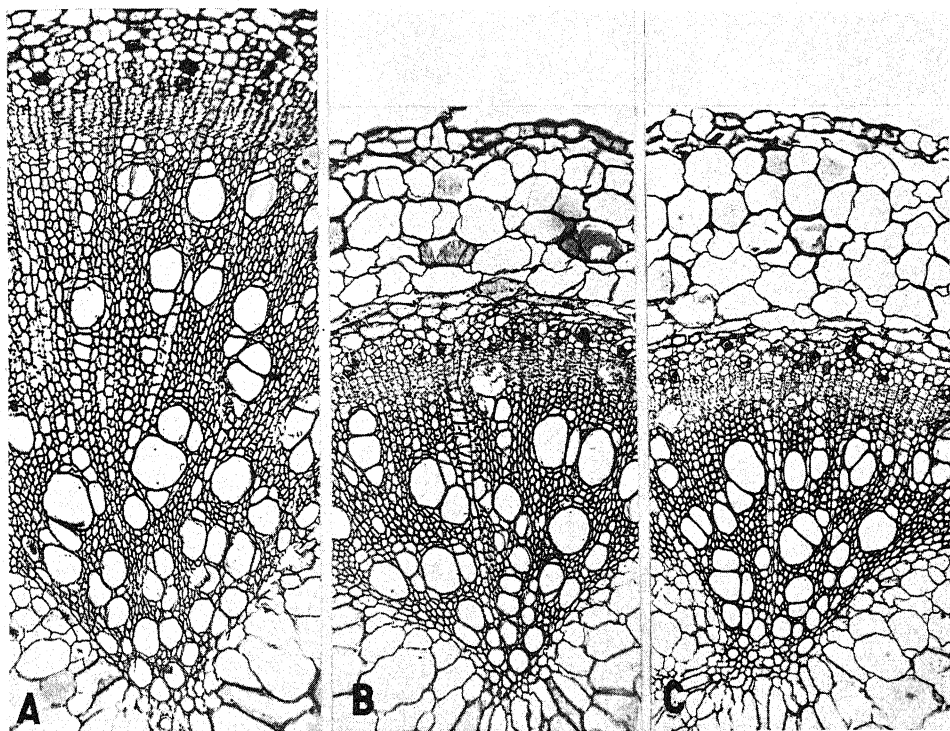


FIG. 6.—Experiment II. Base nutrient series. Transections of basal internodes showing progressive growth inhibition with increasing concentration of culture solution. Sector of largest vascular bundle shown in each case. Osmotic concentrations in atmospheres: A, 0.5; B, 4.5; and C, 6.

sues of the cortex and pith. Measurements were taken of the width of cortex, radial dimension of the largest bundle, and width of the fascicular xylem, outer phloem, and interfascicular vascular tissue. The largest bundle in each transection was measured, as it was determined that its size constituted a reliable index of the total amount of fascicular tissue.

**BASE NUTRIENT SERIES.**—In both experiments there was a marked decrease in the amount of secondary vascular tissue at the high concentrations as compared with the 0.5 and 1.5 atmosphere cultures (fig. 6). Development of interfascicular vascular tissue followed the same general trend as that of the bundles. The reduc-

tion in the secondary (outer) phloem was proportionately greater than that of the xylem. The inner phloem was not measured, since little differential growth response occurred in the primary tissues (table 8).

TABLE 8  
EXPERIMENTS I AND II: GROWTH MEASUREMENTS OF BASAL STEM\*  
(IN MICRONS)

CONCENTRATION OF SOLUTIONS (ATMOSPHERES)	LARGEST BUNDLE		XYLEM		OUTER PHLOEM		INTERFASCICULAR VASCULAR TISSUE	
	I	II	I	II	I	II	I	II
BASE NUTRIENT SERIES								
0.5.....	1910	1550	1550	1190	284	334	357	471
1.5.....	1900	1380	1610	1130	246	215	405	319
3.0.....	1530	1140	1250	968	191	152	349	258
4.5.....	1260	872	1070	723	136	114	243	212
6.0.....	.....	633	.....	532	.....	101	.....	117
SODIUM CHLORIDE SERIES								
1.5.....	1900	1250	1610	1040	232	171	440	266
3.0.....	1600	942	1310	794	205	129	326	180
4.5.....	912	912	729	771	106	110	167	167
6.0.....	.....	765	.....	627	.....	106	.....	129
SODIUM SULPHATE SERIES								
1.5.....	1650	1160	1350	934	223	197	395	238
3.0.....	1410	881	1120	714	182	136	326	238
4.5.....	737	471	592	380	98	60	174	115
6.0.....	.....	288	.....	259	.....	26	.....	80

\* Radial dimensions, averages of four stems in each culture.

The rate of cambial activity was slightly higher at the 1.5 atmosphere level than at the 0.5, but was depressed at the high concentrations. As compared with the 1.5 atmosphere culture, the reduction in cambial rate at the 4.5 and 6 atmosphere levels was 25 per cent and 39 per cent, respectively. The total growth depression was a result of reduction in cell size as well as in number of cells formed. This cellular response was indicated by the decrease in the size of the xylem vessels at the high concentrations (table 9).

There was a marked decrease in the total amount of parenchyma in plants grown in the more concentrated culture solutions. With few exceptions, the per-

centage of medullary tissue increased with increasing osmotic concentration of the culture, while there was little change in the percentage of cortical tissue (table 10).

The differences in the areas of pith may be attributed mainly to variation in the size of the cells, which were larger in stems grown at the lower levels of concentration. The same condition prevailed in the cortex, but in some cases the tangential enlargement of the cortical cells was accompanied by radial divisions.

The collenchymatous cells of plants grown in the 4.5 and 6 atmosphere solutions were smaller than those in the less concentrated cultures, and the thickening

TABLE 9  
EXPERIMENTS I AND II: ALL SERIES. MAXIMUM RADIAL DIMENSIONS  
OF SECONDARY XYLEM VESSELS (IN MICRONS)

CONCENTRATION OF SOLUTIONS (ATMOSPHERES)	BASE NUTRIENT		SODIUM CHLORIDE		SODIUM SULPHATE	
	I	II	I	II	I	II
0.5.....	150	126	.....	.....	.....	.....
1.5.....	152	139	141	132	133	126
3.0.....	126	123	135	114	99	89
4.5.....	117	109	108	92	69	45
6.0.....	.....	91	.....	85	.....	29

of the walls at the angles of the cells was more pronounced. The pericyclic fibers in plants grown at the higher osmotic concentrations were smaller in caliber and their secondary walls were proportionately thicker in comparison with the lumina of the cells.

**SODIUM CHLORIDE SERIES.**—As in the base nutrient series, there were significant reductions in the diameters of the stems grown in culture solutions with high osmotic concentrations as compared with those grown at the 1.5 atmosphere and control (0.5 atmosphere) levels (table 4).

The effect of high salt concentration on the development of secondary xylem and phloem was most pronounced in the more vigorously growing plants of experiment I; but in both experiments there was marked reduction in the amount of secondary fascicular xylem and phloem and in the development of interfascicular vascular tissue (fig. 7; table 8).

In this series, using the 1.5 atmosphere culture as a basis of comparison, the cambial activity was reduced approximately 25 per cent in the 4.5 and 34 per cent in the 6 atmosphere solutions. The effect of high salt concentration on the secondary xylem tissue was evidenced also by the reduction in the size of the vessels and increase in the thickness of their walls (table 9). The reduction in the amount of cortical and medullary parenchyma produced at the high levels of salt concentra-

tion was proportional to the reduction in the size of the stem. There was little variation in the percentage of the total area comprising the cortical and medullary parenchyma (table 10). The differentiation of collenchyma and pericyclic fibers was similar to that in the base nutrient series, but the thickness of the walls was more pronounced at the higher levels.

TABLE 10

EXPERIMENTS I AND II: TRANSECTIONAL AREAS OF STEM AT FIRST INTERNODE\*

CONCENTRATION OF SOLUTIONS (ATMOSPHERES)	TOTAL AREA (SQ. MM.)		PERCENTAGE OF TOTAL AREA			
			CORTEX		PITH	
	I	II	I	II	I	II
BASE NUTRIENT SERIES						
0.5.....	84	50	19	28	46	35
1.5.....	76	53	16	21	45	46
3.0.....	61	44	14	20	50	50
4.5.....	41	35	16	20	50	53
6.0.....	.....	28	.....	21	.....	57
SODIUM CHLORIDE SERIES						
1.5.....	74	50	18	22	44	52
3.0.....	63	36	18	21	47	53
4.5.....	44	33	17	21	58	51
6.0.....	.....	23	.....	28	.....	46
SODIUM SULPHATE SERIES						
1.5.....	63	44	19	20	45	50
3.0.....	41	28	21	26	41	45
4.5.....	15	8	25	43	43	33
6.0.....	.....	3	.....	64	.....	16

\* Averages of four stems in each culture.

**SODIUM SULPHATE SERIES.**—The growth responses followed the same general pattern observed in the base nutrient and sodium chloride series, but the amount of growth depression was greater in this series at corresponding osmotic concentrations (table 4). At equal osmotic concentrations the total amount of vascular tissue was less in the sodium sulphate series than in the sodium chloride series (fig. 8). The reduction in the amount of secondary phloem was greater than that of secondary xylem, and this difference was more pronounced than in the base nu-

trient series. In the sodium sulphate series the amount of growth reduction was much greater between the 3 and 4.5 atmosphere levels than between the 1.5 and 3 atmosphere cultures (table 8).

The rate of cambial activity was much depressed at the 4.5 atmosphere level, being approximately 40 per cent less than that at the 1.5. In the 6 atmosphere culture there was no evidence of interfascicular activity in some of the stems, and the

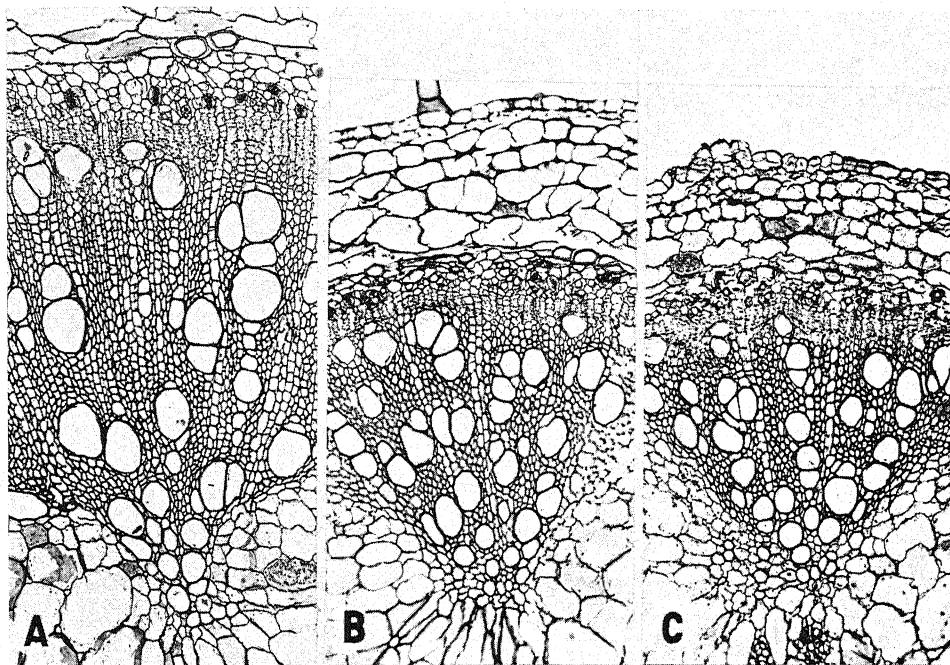


FIG. 7.—Experiment II. Sodium chloride series. Transections of basal internodes showing progressive growth inhibition with increasing concentration of culture solution. Sector of largest vascular bundle shown in each case. Osmotic concentrations in atmospheres: A, 1.5; B, 4.5; and C, 6.

rate of activity of the fascicular cambium was about 60 per cent less than in the 1.5 atmosphere culture. As in the preceding series, there was a reduction in the size of the secondary xylem vessels and a relative increase in the thickness of the cell walls at the high concentrations (table 9).

In experiment I the proportion of cortical and medullary tissues to total trans-sectional area increased slightly with increasing concentration, except at one level. In experiment II the percentage of cortical parenchyma increased markedly, while the medullary tissue decreased. The percentage of the total area comprised of parenchymatous tissue increased at the high concentrations, although the absolute amounts were much decreased (table 10).



There was pronounced accumulation of starch in the parenchymatous cells at the high concentrations. At the 4.5 and 6 atmosphere levels the cells of the endodermis and the pith cells adjacent to the vascular bundles were filled with starch grains. The cortical parenchyma and ray cells also contained more starch than at equivalent concentrations in either of the other series (fig. 8). At the high concentrations the cells of the collenchyma and the pericyclic fibers were smaller and the walls thicker in proportion to the size of the lumina.

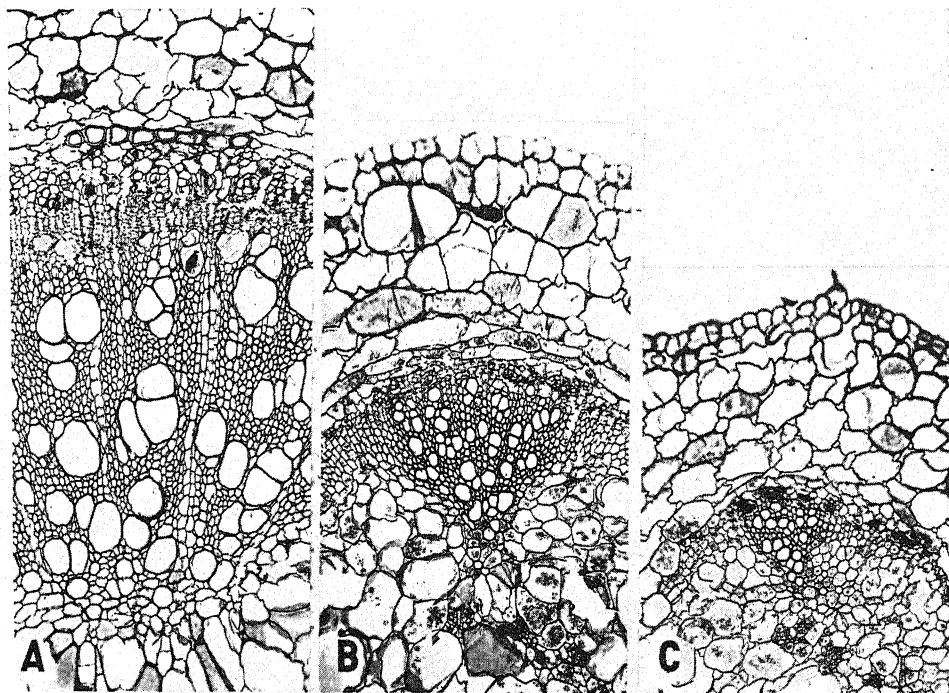


FIG. 8.—Experiment II. Sodium sulphate series. Transections of basal internodes showing progressive growth inhibition with increasing concentration of culture solution. Pronounced accumulation of starch indicated in *B* and *C*. Largest bundle shown in each case. Osmotic concentrations in atmospheres: *A*, 1.5; *B*, 4.5; and *C*, 6.

#### LEAF ANALYSIS

The blade of the leaflet is commonly thin, and the mesophyll consists of a single row of palisade cells and a spongy parenchyma of four or five layers of loosely arranged cells. The main vein projects prominently on the lower surface (fig. 9), and its bicollateral bundle is reinforced by abaxial and adaxial strands of collenchyma (5).

Transections of leaves were examined to determine the growth responses of the vascular tissue of the main vein and the tissues of the blade. The leaves were selected from the final harvest of experiment I and the first harvest of experi-

ment II. Leaves from the final harvest of experiment II were also examined, but these were not strictly comparable with those of the other two harvests, since they were 4 weeks older and had been subjected to less favorable environmental conditions during the additional period of growth. In all series, leaves from the 1.5 and 4.5 atmosphere cultures were examined, and additional studies were made from the 0.5 atmosphere culture in the base nutrient series and the 6 atmosphere cultures of experiment II.

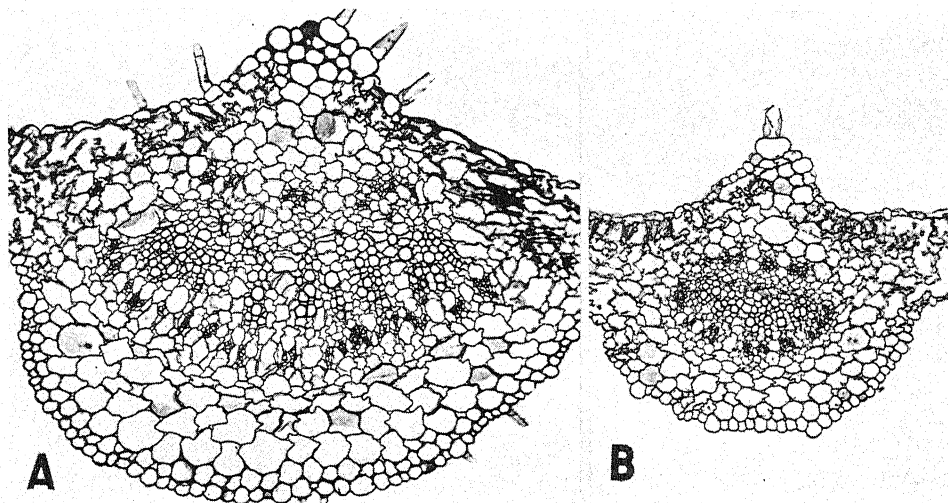


FIG. 9.—Experiment II. Sodium sulphate series. Transections of midribs of terminal leaflets showing growth inhibition at higher concentration of culture solution. Atmospheres osmotic concentration: A, 1.5; B, 4.5.

Representative leaves selected from the median portion of the plant axis were used for biometrical analysis. The height of the vascular bundle of the midrib of the terminal leaflet was used as an index of the amount of vascular tissue supplying the leaves. It provided a suitable basis of comparison with the radial dimensions of the vascular bundles of the stem. The average thickness of the lamina, the height of the palisade layer, and the thickness of the spongy parenchyma were also determined.

**BASE NUTRIENT SERIES.**—In this series the height of the vascular bundle of the main vein decreased at each successively higher concentration of the culture solution, resembling the growth depression of the bundles of the stem in this regard. In experiment II comparison of leaves from the 1.5 and 4.5 atmosphere cultures indicated a significant reduction in the thickness of the leaf of the latter, while in experiment I an increase occurred (table 11).

SODIUM CHLORIDE SERIES.—In both experiments there was a decrease in the vascular tissue of the midvein at the 4.5 as compared with the 1.5 atmosphere culture, and further growth depression was indicated at the 6 atmosphere level in experiment II. There was a significant increase in the thickness of the blade at the higher concentrations. The leaves were about 9 per cent thicker in the 4.5

TABLE 11  
EXPERIMENTS I AND II: LEAF MEASUREMENTS (IN MICRONS)

CONCENTRATION OF SOLUTIONS (ATMOSPHERES)	MAIN BUNDLE HEIGHT		LAMINA THICKNESS		PALISADE HEIGHT		SPONGY PARENCHYMA THICKNESS	
	I	II	I	II	I	II	I	II
BASE NUTRIENT SERIES								
0.5.....	.....	273	.....	187	.....	70	.....	92
1.5.....	360	237	158	177	61	57	73	84
4.5.....	253	223	174	151	61	56	87	72
6.0.....	.....	174	.....	139	.....	58	.....	64
SODIUM CHLORIDE SERIES								
1.5.....	.....	.....	.....	.....	.....	.....	.....	.....
4.5.....	314	246	176	156	67	58	87	70
6.0.....	228	190	192	170	71	68	101	74
.....	.....	177	.....	181	.....	76	.....	82
SODIUM SULPHATE SERIES								
1.5.....	.....	.....	.....	.....	.....	.....	.....	.....
4.5.....	311	266	158	163	48	58	83	73
6.0.....	228	145	206	175	62	66	116	85
.....	.....	148	.....	192	.....	69	.....	93

than in the 1.5 atmosphere cultures; and in experiment II those from the 6 atmosphere culture were 16 per cent thicker than leaves grown at 1.5 (table 11). Leaves from the final harvest of experiment II did not exhibit growth responses similar to those just recorded. The leaves grown at the 4.5 and 6 atmosphere levels were much thicker than those of the 1.5, but the leaves of the plants grown at 6 atmospheres were thinner than those of the 4.5.

SODIUM SULPHATE SERIES.—In both experiments there was a pronounced decrease in the size of the main bundle at the high concentrations (fig. 9). As compared with the 1.5 atmosphere culture, the growth at 6 was depressed about 45 per cent in the sodium sulphate series and 28 per cent in the sodium chloride.

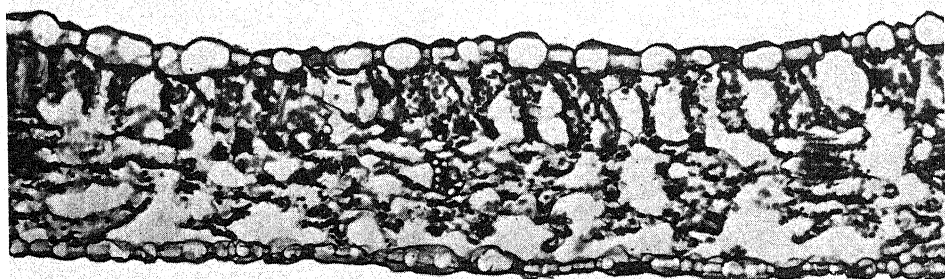
There were significant increases in the thickness of the leaves at the 4.5 and 6 atmosphere levels, ranging from 9 per cent to 30 per cent. At the 4.5 atmosphere level the leaves of the sodium sulphate series were slightly thicker than those of the sodium chloride culture (table 11). At the high levels of salt concentration the relative density of the mesophyll tissues in the sodium chloride and sodium sulphate series appeared to be correlated with the degree of succulence. The lower percentage of dry matter in the tops of the sodium chloride plants as compared with those of the sodium sulphate and base nutrient series at equivalent concentration levels has been noted. In the leaves of plants grown in the high sodium sulphate cultures the palisade layer was much more compactly organized than in the leaves of the corresponding sodium chloride cultures (fig. 10).

### Discussion

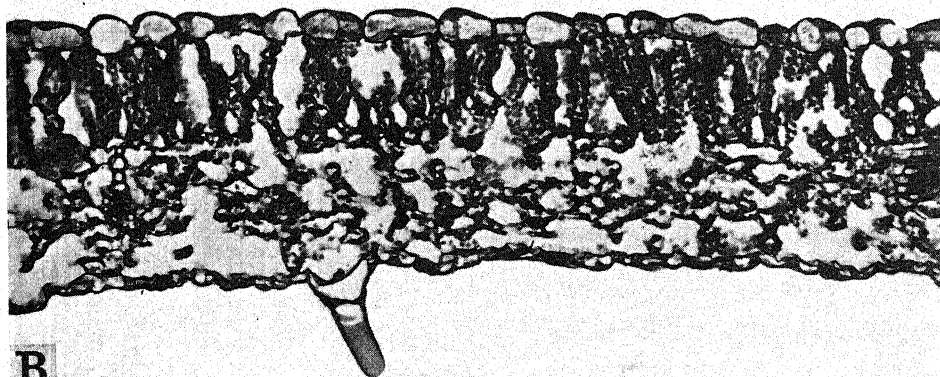
The significance of high concentrations of the soil solution in limiting the intake of water and soluble salts has been pointed out by MAXIMOV (8). BREAZEALE (1), growing wheat in solutions in which the concentration of nutrient salts ranged from 15 to 1550 p.p.m., concluded that the optimum concentration was approximately 155 p.p.m. SHIVE (12), using a three-salt solution, obtained best results at 1.75 atmosphere osmotic concentration, poorest ones at 0.1 atmosphere, and intermediate effects at 4. HOAGLAND (6) has defined optimum total concentration of a nutrient solution as "the least concentration giving a yield equal to any higher concentration." On that basis he found the optimum concentration to be 0.6 atmosphere and inhibitive concentrations at 2-2.5 atmospheres. TRELEASE (13) obtained optimum yields at 1.6 atmosphere, and found decreasing dry weight of tops with increasing concentration. NIGHTINGALE and FARNHAM (11) obtained best results with sweet peas at 0.5-1 atmosphere osmotic concentration.

In the present experiments with nutrient cultures at osmotic concentrations of 0.5, 1.5, 3, 4.5, and 6 atmospheres, maximum vegetative growth was obtained at 1.5 and a pronounced growth depression was noted at higher concentrations. No cultures were maintained at 1 atmosphere, and it is possible that for the solution used the optimum concentration as defined by HOAGLAND may lie somewhere between 0.5 and 1.5 atmospheres.

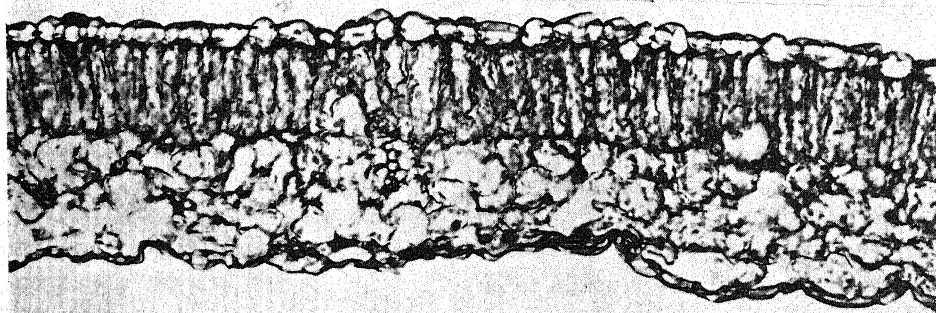
In cases where the high osmotic concentrations were obtained by the addition of sodium chloride or sodium sulphate to the base nutrient solution (0.5 atmosphere concentration), the growth depression at isosmotic concentrations was greater than in the base nutrient cultures. Comparisons of fresh and dry weights, gross morphology, and detailed anatomy indicated that the plants in the high sodium sulphate cultures were smaller than those in the corresponding cultures in the sodium chloride series. This suggests that there is an ionic influence which operates in addition to the effect of total salt concentration. EATON (3) has indicated



**A**



**B**



**C**

FIG. 10.—Transsections of blades of terminal leaflets showing relative organization of palisade and spongy parenchyma: *A*, base nutrient culture, 1.5 atmosphere; *B*, sodium chloride culture, 4.5 atmospheres; *C*, sodium sulphate culture, 6 atmospheres.

a tendency for sulphate to be about half as toxic as chloride on a milliequivalent basis, but notes that "the tomato is out of line" in this regard.

In the sodium chloride series the percentage of dry matter increased very slightly or not at all with increasing concentration, while in the base nutrient series—and to a greater degree in the sodium sulphate series—there were marked increases in the percentage of dry matter, indicating a greater degree of succulence in the high chloride series.

The succulence of the leaves in the sodium chloride series corresponded with that observed by WUHRMANN (16), who found that the thickness and degree of succulence of the leaves of *Lepidium sativum* and *Nicotiana* could be modified by the addition of sodium chloride to nutrient solutions. VAN EIJK (14) investigated the effect of sodium chloride on the halophyte *Salicornia herbacea* and found optimum development with a 4/12 molar solution containing 2 per cent sodium chloride. He attributed the growth responses to the chloride ion rather than to the osmotic value of the solution or the concentration of the sodium ion, and concluded that cations do not affect succulence and that it is not induced by sulphate or nitrate ions. It seems possible, however, that the sodium ion may have an inhibitory effect on growth. At equal osmotic concentrations the solutions in the sodium sulphate series had a higher concentration of sodium than the sodium chloride cultures, and the accumulation of sodium by the plants showed a close relationship to the amount of sodium present in the solution, irrespective of the anion.

Growth depression may be the result of environmental factors which restrict the size of cells by accelerating their rate of maturation, or which inhibit the rate of activity of primary or secondary meristems. In the stems examined the number of primary cells at any given transectional level did not appear to be materially reduced by high salt concentration. The cells of the fundamental parenchyma were smaller, but the elements of the primary vascular tissue did not exhibit significant variation in size. The cells derived from the secondary meristems, especially the xylem elements, were smaller in size at the high concentrations.

In all series there was less cambial activity in plants grown in culture solutions with high osmotic concentrations than in those at the 1.5 atmosphere levels. Comparisons of cambial rate indicated that, at isosmotic concentrations, the rate was greatest in the base nutrient series, least in the sodium sulphate, and intermediate in the sodium chloride series. Between the 1.5 and 4.5 atmosphere levels the reduction in cambial rate ranged from 25 per cent to 42 per cent.

Starch accumulation may result from a number of factors. NIGHTINGALE (9) and FOSTER and TATMAN (4) have indicated the influence of temperature in this regard, and the latter have also called attention to the effects of variation in soil moisture. A number of investigators have shown that the carbohydrate metabo-

lism of the tomato plant is influenced by the supply of nutrient constituents, including ammonium, nitrate nitrogen, and potassium (7, 10, 15). Although no quantitative data were obtained, pronounced accumulation of starch in the storage parenchyma of the basal portion of the stem appeared to be correlated with high salt concentration. This was most evident in the high sodium sulphate cultures, in which the medullary, ray, and cortical parenchyma and the endodermal cells contained large reserves of starch.

### Summary

Tomato plants were grown in three series of culture solutions: (a) a base nutrient series consisting of cultures adjusted to 0.5, 1.5, 3, 4.5, and 6 atmospheres osmotic concentration; (b) a sodium chloride series consisting of cultures containing base nutrient (0.5 atmosphere) plus sodium chloride to adjust the concentrations to 1.5, 3, 4.5, and 6 atmospheres osmotic concentration, respectively; and (c) a sodium sulphate series set up as in (b) but with sodium sulphate as the added salt instead of sodium chloride.

1. The concentration of the culture solution exerts an important influence on growth. In all series the growth of the plants as measured by height, diameter of stem, and dry weight was less at the high concentrations than at the control or 1.5 atmosphere levels. The greatest differences occurred in the sodium sulphate and the smallest in the base nutrient series.

2. The smaller diameters of stems at the high levels of salt concentration were correlated with significant differential reductions in the tissue systems. In general, the reduction of the vascular tissues, on the basis of percentage of total area, was greater than that of the parenchymatous tissue of the cortex and pith.

3. The reduction in the amount of vascular tissue at the high levels of salt concentration was related to more rapid maturation and consequent reduction in the size of the cells. The secondary xylem vessels were smaller in diameter at the high concentrations in all series, and the walls were thicker in proportion to the lumina of the vessel segments.

4. In the base nutrient and sodium sulphate series the proportion of secondary phloem to xylem was decreased at the high levels of salt concentration. In the sodium chloride series the proportionate amounts of secondary xylem and phloem remained approximately the same at all levels.

5. In all series the cells of the mechanical tissues were smaller in caliber and thicker walled in plants grown at high osmotic concentrations.

6. Cambial activity was inhibited by high concentrations of salts in all series. At isosmotic concentrations the cambial rate was greatest in the base nutrient and least in the sodium sulphate cultures.

7. The leaflets of plants grown at the high salt concentrations were significantly thicker in the sodium chloride and sodium sulphate series than in the base nutrient cultures.

8. The leaves of plants grown in high sodium chloride solutions were more succulent than those of the base nutrient and sodium sulphate series. This was correlated with the loose arrangement of the tissues of the mesophyll. In contrast, the palisade tissue in the leaves of plants grown in high sodium sulphate cultures was very compact.

9. The greater succulence of plants grown in high sodium chloride solutions was also indicated by the percentage of dry matter of tops. This remained essentially constant at all levels of concentration in the sodium chloride series, while it increased at the high concentrations in the base nutrient and sodium sulphate cultures.

10. Flower bud formation was retarded and probably reduced on plants grown in high sodium chloride solutions, and anthesis was delayed. There was a more pronounced effect in the high sodium sulphate cultures at the 4.5 atmosphere level, and no evident flower buds had developed up to the time of harvest.

11. High starch accumulation in the parenchymatous tissues of the basal stem was apparently correlated with high salt concentration in the culture solutions. This was most pronounced in the sodium sulphate cultures at 4.5 and 6 atmospheres osmotic concentration, but was also marked in the stems of plants grown at high sodium chloride levels.

12. Osmotic concentration of the sap increased in all series with increasing concentration of the culture solution.

13. With increasing concentration of the culture solutions there was increased accumulation of sodium and chloride ions in the tops of plants in the sodium chloride series. In the sodium sulphate series accumulation of the sodium ion increased as the concentration of the culture solution increased, but there was less correlation between the accumulation of the sulphate ion and the concentration of sulphate in the solutions.

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# INFLUENCE OF PREPARATIVE PROCEDURE ON THE PURITY OF CHLOROPHYLL COMPONENTS AS SHOWN BY ABSORPTION SPECTRA

F. P. ZSCHEILE AND C. L. COMAR<sup>1</sup>

(WITH FOUR FIGURES)

## Introduction

The lack of agreement on the quantitative expression of the absorption spectra of chlorophylls *a* and *b* has been the source of considerable confusion. This has been ably discussed recently by MACKINNEY (7). The causes of these difficulties may be broadly classified into two groups, those resulting from the use of different spectro-photometric methods and those arising from the presence of accompanying impurities or decomposition products. As will be shown later, the latter group is perhaps of more importance because of the extreme lability of isolated chlorophyll. The high sensitivity of the spectro-photoelectric method makes it possible to detect changes which cannot be followed by other methods. The establishment of accurate absorption values for the individual chlorophyll components is essential for quantitative treatment of the relations of light to chlorophyll systems, as in photosynthesis, and is indispensable for spectroscopic analysis of pigment mixtures.

While reinvestigating the absorption spectra of chlorophylls *a* and *b* in ether solution, it was observed that significant changes were caused by variations in preparative procedure. Further study of these changes led to certain modifications of existing methods which seemed necessary to avoid decomposition. The present method will be described in some detail, because neglect of certain precautions—one of which has not been observed in previously described methods for pigment isolation—may lead to an amount of decomposition which may alter the absorption values considerably. Such slight changes in composition cannot be followed reliably by elementary analysis.

## Experimentation

Numerous variations in procedure have been tried and the following method has been found to yield chlorophyll components with consistent spectroscopic properties. Certain general features of the WILLSTÄTTER-STOLL (10) method have been retained. Both components are separated and purified by adsorption.

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## PREPARATION OF CHLOROPHYLL A

A semi-darkened room is desirable for all preparative procedures. About 1 kg. of clean fresh green leaves is disintegrated in a Waring Blendor (1), in the presence of cool acetone (about 20° C.) to which a small amount of calcium carbonate has been added. One hundred gm. of leaf material is conveniently extracted in 400 cc. of acetone; usually 3-5 minutes in the Blendor per batch is sufficient for preparative extraction. These conditions may be varied somewhat, depending on the type of material used and the degree of extraction desired. Usually one extraction leaves the cell fragments almost colorless after filtration and washing.

The mixture is filtered by suction and the resulting acetone extract is added carefully to a solution containing 1 liter of petroleum ether<sup>2</sup> and 100 cc. of acetone in a 4-liter glass separatory funnel. The filtrate from each batch is added individually. After each third addition, the solution is rotated gently and distilled water carefully added until two distinct layers are formed. (It is advisable that a small amount of calcium carbonate be added to all distilled water used for washing purposes.) Most of the acetone and water-soluble impurities are removed in the aqueous layer, which is discarded before further addition of acetone filtrates.

The petroleum ether solution is scrubbed through distilled water. This is done by allowing the solution to flow by gravity through a narrow tube submerged 5-10 cm. below the surface of the water (7), contained in a 4-liter separatory funnel. The solution is then washed five or more times with 200 cc. of 80% methyl alcohol. Before use, the methyl alcohol is saturated with petroleum ether and 0.01 gm. of oxalic acid per liter added to prevent allomerization. The last washing should be a light greenish yellow. The solution is now scrubbed through distilled water until precipitation of the chlorophyll occurs; three or four washings usually suffice and the time involved in this step is 1-2 hours.

The chlorophyll suspension is dried over anhydrous sodium sulphate and filtered on 3-cm. layer of powdered sucrose (ordinary powdered sugar<sup>3</sup> is used). This layer is made on a Buchner funnel 14 cm. in diameter and 5.5 cm. deep. The sucrose is wet with petroleum ether and suction applied; the sugar is pressed down and, when dry, rubbed to form a smooth hard surface. The precipitated pigment is washed exhaustively with petroleum ether to remove carotenoids. The pigment layer is mechanically removed, while still moist, and extracted with a maximum of 60 cc. of ether.<sup>4</sup> The resulting solution contains crude chlorophyll. This step requires 30-50 minutes.

The ether solution of crude chlorophyll is made up to contain 70% petroleum ether and 30% ether. This solution is adsorbed on sucrose, the column dimensions being 5 cm. in diameter and about 35 cm. in length. When used for adsorption,

<sup>2</sup> All petroleum ether used had a b.p. of 35°-45° C.

<sup>3</sup> C and H confectioner's powdered sugar (contains 3% starch).

<sup>4</sup> Ether designates diethyl ether.

the powdered sugar is first dried at 90° C. in vacuum and stored in air-tight bottles. Under normal conditions only two zones are observed: a green band 4-10 cm. in length and below it a contiguous blue zone 2-6 cm. in length. The time required for this separation is only about 40 minutes, since it has not been found necessary to develop the chromatogram by washing. The green layer is mechanically removed and may be used for the subsequent isolation of chlorophyll *b*, although it was found more convenient to make a separate extraction for the preparation of the *b* component. The walls of the column are then cleaned with ether-soaked cotton to avoid any minute contamination of the chlorophyll *a* with the *b* component. The center half of the blue layer is removed and immediately stirred into ether, the top and bottom portions being discarded. The resulting suspension is filtered on a Buchner funnel and the pigment thus eluted. The ether solution is washed thoroughly with distilled water and dried over sodium sulphate. The solution is filtered through a fine sintered glass plate, and the pigment is then ready for spectroscopic study. The time required for this entire preparation should not exceed 5-6 hours.

#### PREPARATION OF CHLOROPHYLL B

The procedure described for chlorophyll *a* is followed in detail up to and including the adsorption of the pigments on sucrose. In the isolation of the *b* component, however, it is necessary to wash the column exhaustively; 1-1.5 liters of fresh solvent (70% petroleum ether, 30% ether) are used. After the washing, the column consists of a 5-7 cm. layer of colorless adsorbent, below which is a 23-27 cm. green zone, which in turn is followed by the blue band of component *a*. The center portion of the green zone is mechanically removed, taking all precautions to avoid contamination, and is stirred immediately into ether, filtered, and eluted.

This solution is made up to contain 70% petroleum ether and 30% ether and is reabsorbed on sucrose. After washing as indicated, the green zone is usually separated from the blue by about 1 cm. of colorless adsorbent. The center portion of the green zone is again removed and the pigment eluted. The pigment solution is again adjusted to a concentration of 70% petroleum ether and adsorbed a third time. This chromatogram should consist of a single homogeneous zone. The final pigment solution is prepared in precisely the manner described for chlorophyll *a*. Since each column requires about 2 hours, the total time required for the isolation of the *b* component from plant material is 11-13 hours. Ordinarily one worker will require 2 working days for this preparation and for spectroscopic observations. Ether solutions may be kept over night at 0° C.

The solvents were purified as described by ZSCHEILE (13). In some cases the ether was purified by distillation after standing over solid potassium hydroxide. The spectroscopic results were the same as when sodium was used.

## ABSORPTION METHODS

The absorption spectra were determined by a spectro-photoelectric method similar to that described by HOGNESS, ZSCHEILE, and SIDWELL (4). The most important difference is that in our optical system a Large Müller-Hilger Universal Double Monochromator with crystal quartz optics is employed instead of a Zeiss Fixed-arm Spectroscope (with deviation of  $90^\circ$ ) with glass optics. A figured crystal quartz lens, corrected for spherical aberration, is used instead of the simple biconvex crystal quartz condensing lens 1 (4, fig. 2). A crystal quartz field lens is mounted directly in front of slit 1. No other lenses are used to focus the source radiation upon the slit. Otherwise the two sets of equipment are very similar, except for certain changes made for greater convenience or stability. The entire set-up will be described in full at a later time.

All three slits of the double monochromator were maintained at equal widths and the exit slit was confined to a length of 6 mm. by a diaphragm. The dispersion of this instrument with quartz optics is almost equal to that of the Zeiss instrument with glass optics from 4000–5600 Å and is greater from 5600–7000 Å. The dispersion, expressed in Å per millimeter, is as follows: 63 at 4000 Å, 168 at 5500 Å, and 290 at 7000 Å. Measurements were made at intervals of 20–40 Å over most of the spectrum and at 10-Å intervals over the higher maxima. The region from 3800 to 6900 Å was studied.

The cell<sup>5</sup> length was 1.0003 cm., and the concentration was adjusted to keep the  $\log_{10} \frac{I_0}{I}$  values between 0.200 and 0.800. The specific absorption coefficient  $\alpha$  is defined as follows:

$$\alpha = \frac{\log_{10} \frac{I_0}{I}}{cl}.$$

$I_0$  = intensity of light transmitted by solvent-filled cell.

$I$  = intensity of light transmitted by solution-filled cell.

$c$  = concentration in gm. per liter.

$l$  = thickness of solution layer in cm.

The following technique is used to obtain the concentration of an ether solution of chlorophyll from a 10-cc. aliquot of that solution. A clean, tared 10-cc. volumetric flask is filled to the mark with the solution. The ether is evaporated under a water pump vacuum, care being taken to avoid spattering. After the ether is removed the flask is kept at  $103^\circ$  C. for an hour, allowed to cool for 30 minutes in a

<sup>5</sup> Fused Pyrex absorption cells (American Instrument Co., Style DX, Class 3 Tolerance) with ground stoppers, were used.

desiccator, and weighed on a microbalance. It is advisable to tare the flask by using pure ether and following the procedure already described. When the concentration is 0.2 gm. per liter or more, the error is  $\pm 0.4\%$  as checked against known solutions prepared from weighed samples of dried chlorophyll. In most cases the average error of three determinations is less than  $\pm 0.3\%$ . For lower concentrations, or when greater accuracy is desired, 25-cc. volumetric flasks may be used.

## Results

### ABSORPTION SPECTRA

The absorption spectra of chlorophylls *a* and *b* in ether solution are presented in figure 1. In figure 2 the regions of low absorption between 4400 and 6400 Å are plotted on an enlarged scale to facilitate later discussion; the accuracy of presentation is then more comparable with that of the measurements. The experimental points are chosen from two or more complete curves of different preparations to produce a representative curve. Tables 1 and 2 present the numerical values of absorption coefficients at maxima and minima for numerous preparations of chlorophyll. Typical values for concentrations and spectral regions isolated are representative of the group but are not all descriptive of the same preparation.

The ultraviolet absorption spectrum has been investigated in a very preliminary way. No detail of special interest was observed.

We have used diethyl ether as a solvent for spectroscopic observations, chiefly because the sucrose from the adsorption column can be completely removed from ether by washing with water. When acetone is used for direct elution from the adsorbent, a small amount of sucrose remains in solution and causes large errors in the weight determination of the chlorophyll. Attempts to correct for sucrose content by the use of acetone saturated with sucrose or by removal of sucrose quantitatively by freezing methods were unsuccessful. Moreover, data from more laboratories are available for ether solutions than for other solvents and we have experienced no difficulty in the use of ether. All the solutions, solvent, and glassware employed for dilutions were maintained at 25° C., and the absorption cell chamber was thermostated at 25° C.

In figures 3 and 4 the spectra of the individual components are compared with those of the products formed from the components by acid. The "pheophytin" was prepared by the addition of approximately 0.1 cc. of concentrated hydrochloric acid to the pure chlorophyll component in approximately 50 cc. of ether solution. The absorption measurements were made after the solution had remained 1–3 days at room temperature in the dark. The concentration was calculated from that of the original pure chlorophyll solution, assuming that two hydrogens replace the magnesium in the molecule. The maxima of these curves agree

# ABSORPTION SPECTRA OF CHLOROPHYLLS A AND B IN ETHER

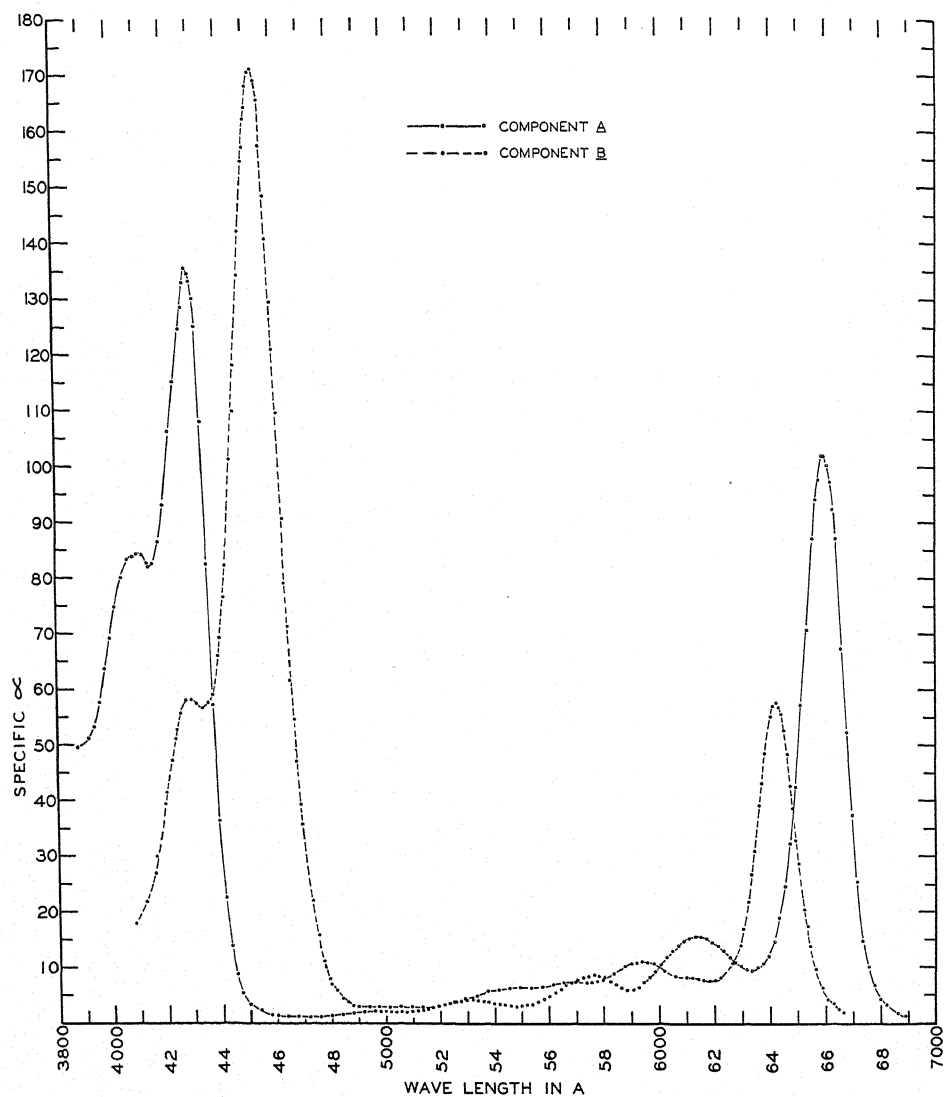


FIG. 1

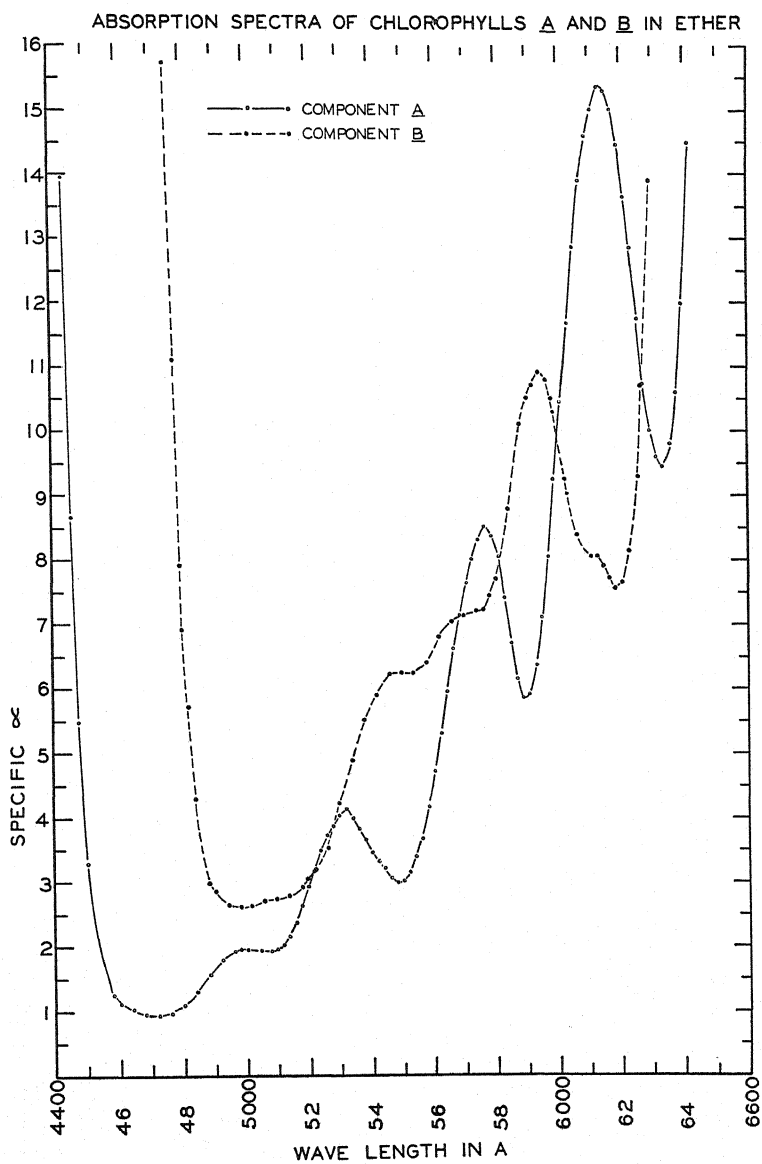


FIG. 2



TABLE 1  
ABSORPTION SPECTRA OF CHLOROPHYLL *a* IN ETHER SOLUTION

WAVE LENGTH Å	SPECIFIC ABSORPTION COEFFICIENTS						AVERAGE	MEAN DEVIATION (%)	TYPICAL CONCENTRATION PER LITER (GM.)	TYPICAL SPECTRAL REGION ISO- LATED (Å) (4)
	1 SPINACH	2 SPINACH	3 CORN	4 SPINACH	5 SPINACH	6 SPINACH				
Maxima:										
6600.....	103.5	101.6	103.5	101.8	100.2	101.8	102.1	0.94	0.003	30
6140.....	15.7	15.3	15.6	15.6	15.4	15.3	15.5	0.98	0.030	26
5760.....	8.44	8.56	8.50	8.56	8.50	8.50	8.51	0.39	0.040	23
5320.....	4.22	4.19	4.18	4.16	4.03	4.03	4.14	1.34	0.120	18
5050 (pheophy- tin <i>a</i> maxi- mum).....	1.96	1.97	1.94	1.93	1.97	1.94	1.95	0.77	0.200	16
4290.....	139†	131	137	138	133	135	135	1.63	0.003	10
4100.....	89.0†	83.8	86.5	85.5	85.7	84.3	85.2	1.03	0.003	9
Minima:										
6330.....	9.32	9.40	9.46	9.80	9.76	9.45	9.53	1.73	0.040	28
5890.....	5.92	5.90	5.76	6.00	6.12	5.85	5.93	1.54	0.050	24
5480.....	2.96	3.05	3.02	3.17	3.01	2.99	3.03	1.67	0.150	20
4720.....	1.60†	0.956	0.928	0.923	0.929	1.27†	0.934	1.18	0.230	13
R <sub>a</sub> *.....	52.8	51.6	53.3	52.7	51.0	52.4	.....	.....	.....	.....

$$* R_a = \frac{a_{6600}}{a_{5050}}.$$

† These values excluded from averages because of carotenoid content.

TABLE 2  
ABSORPTION SPECTRA OF CHLOROPHYLL *b* IN ETHER SOLUTION

WAVE LENGTH Å	SPECIFIC ABSORPTION COEFFICIENTS			AVERAGE	MEAN DEVIATION (%)	TYPICAL CONCENTRA- TION PER LITER (GM.)	TYPICAL SPECTRAL REGION ISO- LATED (Å)
	1 BARLEY	2 SPINACH	3 SPINACH				
Maxima:							
6425.....	57.6	55.4	57.5	56.8	1.71	0.008	38
5940.....	11.1	10.6	10.9	10.9	1.83	0.040	32
5500.....	6.27	6.10	6.24	6.20	1.13	0.040	27
4530.....	173	169	171	171	0.78	0.003	15
4300.....	59.1	58.2	58.1	58.5	0.74	0.008	13
Minima:							
6180.....	7.74	7.54	7.55	7.61	1.14	0.040	35
5200 (pheophy- tin <i>b</i> maxi- mum).....	3.15	3.02	3.07	3.08	1.51	0.180	23
5100.....	2.68	2.64	2.73	2.68	1.12	0.180	22
R <sub>b</sub> *.....	18.3	18.3	18.7	.....	.....	.....	.....

$$* R_b = \frac{a_{6425}}{a_{5200}}.$$

# ABSORPTION SPECTRA IN ETHER

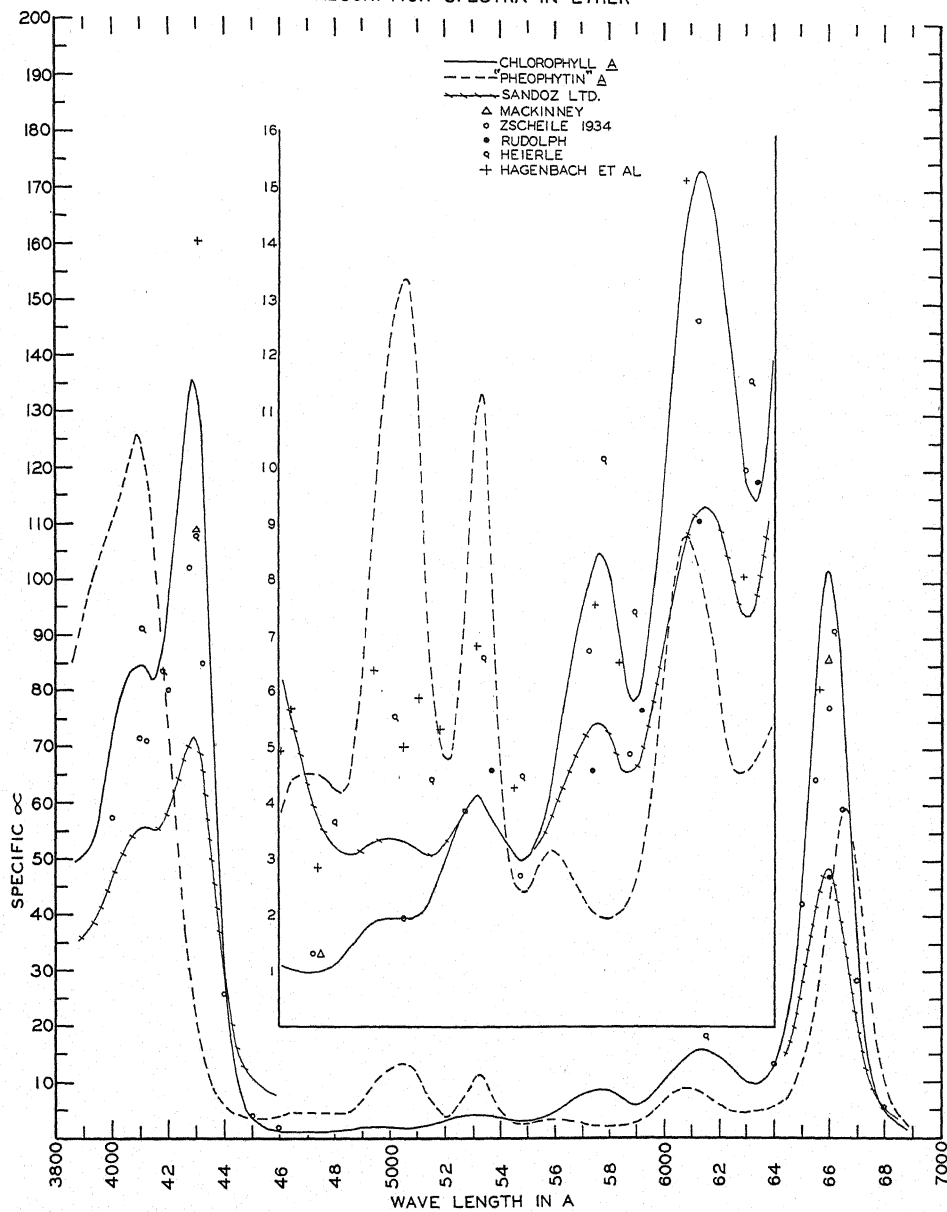


FIG. 3

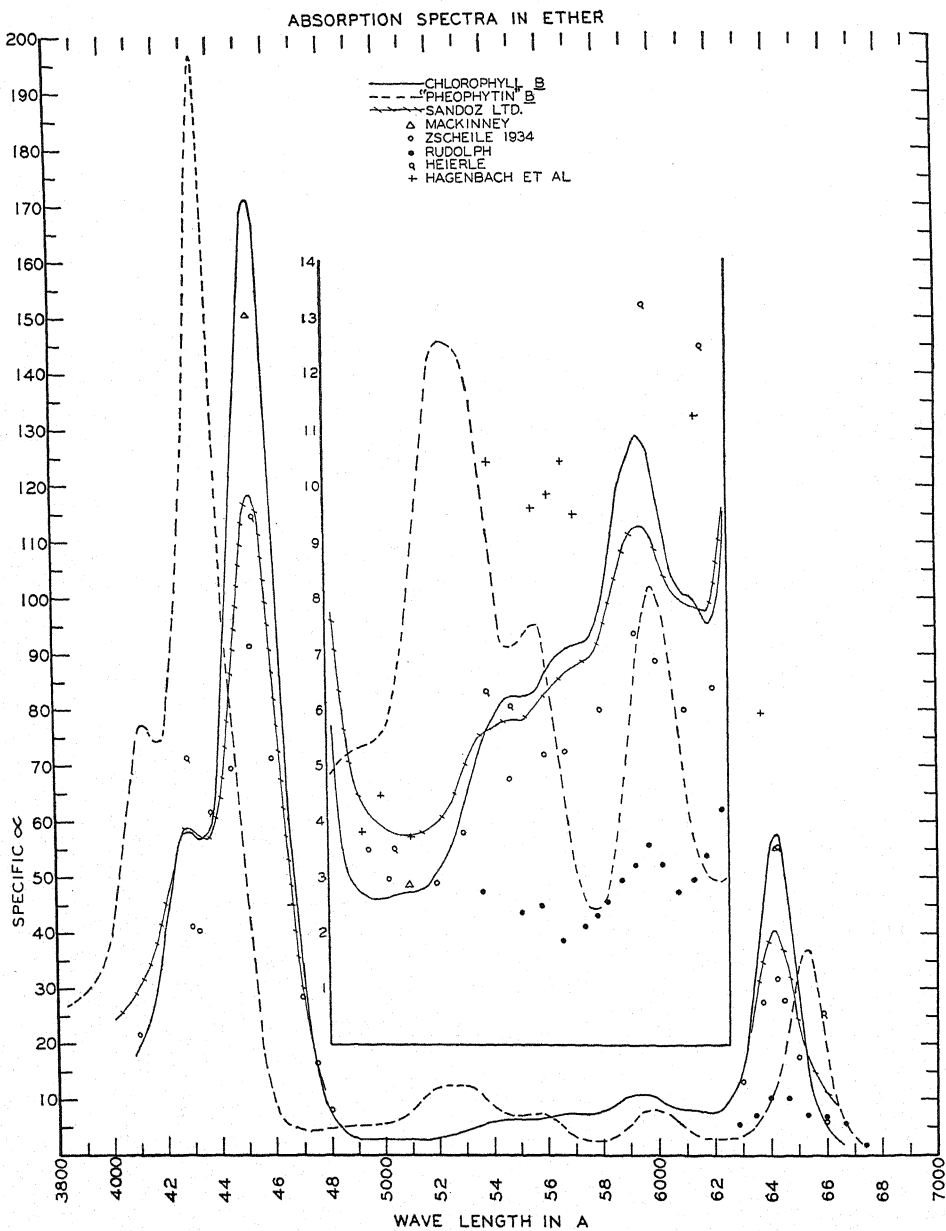


FIG. 4

in wave length with those ascribed to pheophytin in the literature. These curves are not presented as those of pure pheophytin, however, since different preparations had absorption coefficients which differed by 2-15% at certain wave lengths. The most representative curves are plotted. For the purposes of this paper, these differences are unimportant.

Figures 3 and 4 also contain the absorption spectra of chlorophyll components *a* and *b*, which were purchased from Sandoz, Ltd., Basle, Switzerland. They are designated by the manufacturer as "chromatographically pure chlorophyll preparations for scientific purposes," purified by adsorption on sugar. The preparations were made in September, 1939, from the leaves of the stinging nettle (*Urtica dioica*). The spectra were determined on weighed samples in September, 1940. In addition, other absorption values for chlorophyll components in ether solution, which have come to the writers' attention, are included for comparative purposes.

#### SPECTROSCOPIC CRITERIA OF PURITY

Since chlorophyll tends to lose its magnesium relatively easily, it is of interest to study the spectra of pheophytin. It was noted that the absorption values of all our chlorophyll *a* preparations which had undergone small amounts of degradation occurred between the chlorophyll and pheophytin values, especially for wave lengths above 4700 Å. It is evident from the nature of the curves that initial degradation appears to be similar to pheophytin formation. HUBERT's data (5) on the effect of light on chlorophyll also show this trend.

It is clear (figs. 3, 4) that small amounts of pheophytin *a* in chlorophyll *a* may be most easily detected by a study of the spectral regions near 6600 and 5050 Å.

In the case of *b*, the wave lengths are 6425 and 5200 Å. Thus the ratio  $\frac{a_{6600}}{a_{5050}}$  may be used as a sensitive criterion for the usual forms of degradation in chlorophyll *a*.

This ratio will hereafter be designated as  $R_a$ .  $R_b$  is the ratio  $\frac{a_{6425}}{a_{5200}}$ .

In experiments with calcium carbonate as an adsorbent, it was noticed that chlorophyll *a*, which had been dried by evaporation with a water pump and then stored in vacuum over night at 0° C., formed an olive green ether solution instead of the original blue. This was indicative of pheophytin formation, and raised the question as to whether the same thing might not occur when other adsorbents were used, but to a less degree, so that the change could not be observed visually. This change was studied spectroscopically. WILLSTÄTTER and STOLL (10) observed a change of spectroscopic properties when certain chlorophyll derivatives were dried from ether solution. Our samples designated as "dried" were evaporated to dryness in beakers with a water pump and immediately placed in a vacuum of about 20  $\mu$ . All samples were kept at 0-5° C. and not exposed to light. The glass-

ware was washed in acid dichromate solution, followed by tri-sodium phosphate to avoid the possibility of degradation due to residual acid on the walls. The following data are chosen as representative of many experiments showing the same trends.

#### SAMPLE AND TREATMENT

	$R_a$
I. Chlorophyll <i>a</i> prepared by adsorption on calcium carbonate.....	42.3
A. Preparation I stored in solution over night.....	38.7
1. Preparation IA stored in solution over night.....	33.8
2. Preparation IA stored dry over night.....	19.7
II. Chlorophyll <i>a</i> prepared by adsorption on sucrose.....	50.8
A. Preparation II in solution for 8 days.....	49.1
B. Preparation II dry for 8 days.....	46.9
III. Chlorophyll <i>a</i> prepared by adsorption on sucrose.....	52.4
A. Preparation III in solution for 13 days.....	51.3
B. Preparation III dried with oxygen-free nitrogen, allowed no contact with air, and stored for 13 days.....	42.8
IV. Chlorophyll <i>a</i> prepared by adsorption on sucrose and mistreated by drying and storage under laboratory conditions.....	32.9
A. Preparation IV in solution over night.....	33.0
B. Preparation IV dry over night.....	30.1
C. Preparation IV stored dry on powdered glass over night.....	23.4
$R_b$	
V. Chlorophyll <i>b</i> prepared by adsorption on sucrose.....	18.9
A. Preparation V in solution for 5 days.....	17.7
1. Preparation VA in solution for total of 9 days.....	16.6
B. Preparation V dry for 5 days.....	13.9
1. Preparation VB redried and stored for 4 more days.....	4.4

It is evident that the adsorbent may influence the stability of the final solution. The drying effect is independent of the presence of oxygen. Redrying and increased contact with glass surfaces apparently increase the amount of degradation. All samples show a positive phase test and, in the case of chlorophyll *a*, solutions having a ratio as low as 30 cannot be distinguished visually from those with ratios of 51 to 53.

#### OTHER CRITERIA OF PURITY

The phase test was positive on all preparations; the transient color was yellow for component *a* and red for component *b*.

The absence of hydrolytic loss of phytol is indicated by the fact that color was not extracted from ether solutions by 22% HCl. This is confirmed by an analysis for total nitrogen which gave a value of 6.18% nitrogen in chlorophyll *a*. The theoretical value is 6.28%. In no case has difficulty been experienced due to hydrolysis, nor is there any evidence that it had occurred.

## Discussion

### PREPARATION

Exposure of the pigments to light, heat, drying, or acid must be avoided during the preparation. Small amounts of calcium carbonate are added to the acetone used in extraction (to neutralize plant acids which may be liberated) and also to all distilled water used in washing procedures.

The extreme sensitivity of chlorophyll solutions to light is shown by an experiment in which a dilute ether solution of chlorophyll *a* in the absorption cell was exposed to ordinary room light (northern exposure) for 30 minutes. The absorption value at 4290 Å decreased 14% during this time. The original stock solution, kept in the dark 3 hours at room temperature, showed no decrease. This indicates the necessity for elimination of all possible light exposure at all times.

Fresh leaves are used in order to avoid any possible pigment degradation due to drying. Acetone has been found satisfactory for extraction. Ice may be added directly to the mixture in the Waring Blendor to counteract heat generated by the Blendor or effects of excessively warm weather.

The pigments may be transferred to petroleum ether after having been in contact with acetone for only 5–7 minutes; this minimizes the possible loss of phytol due to enzyme action (7). The methyl alcohol washing serves to eliminate most of the xanthophylls and, although it causes considerable loss of chlorophyll *b*, the yield is always sufficient for quantitative spectroscopic studies.

The scrubbing method, suggested by MACKINNEY (7), greatly shortens the time required for precipitation. The chlorophyll suspension is filtered on sucrose rather than talc, since the former may be easily washed out of the ether solution and there is evidence that contact with talc may cause degradation of the pigment. WINTERSTEIN and SCHÖN (12) also observed this cause of degradation. Experiment has shown that a second washing with methyl alcohol, reprecipitation, and rewashing with petroleum ether usually do not increase the purity of the preparation so far as contamination with carotenoids is concerned. It was observed that removal of carotenoids is essential for successful separation of *a* and *b* components on sucrose.

Sucrose was chosen for separation of the components by adsorption because of the following facts: (*a*) we have found that sucrose does not induce pigment degradation, which is not the case for  $\text{CaCO}_3$ ,  $\text{Ca(OH)}_2$ ,  $\text{MgO}$ , and at least for certain brands of talc; (*b*) under our conditions chlorophyll *b* may be obtained as a discrete zone on the second column—MACKINNEY (7) obtained this result with inulin; (*c*) sucrose is readily removed from ether solutions by washing with water; and (*d*) the material is inexpensive and readily available.

When old chlorotic leaves are used, or when pigment degradation has occurred,

a dark layer (probably pheophytin) is found below the *a* zone on the column. When all precautions have been observed, however, no indication of degradation is evident either below the *a* or above the *b* band. According to WILSON (11), the uppermost color zone of a chromatogram contains some of each solute initially present; this has been confirmed by the present observations. Thus in a column in which the zones are contiguous, all except the lowest would be expected to contain more than one substance. If pheophytin formation is avoided, chlorophyll *a* will be the lower zone and there will be no need for exhaustive washing of the chromatogram when this component is desired. The difficulty and uncertainty in the separation of pheophytin *a* from chlorophyll *a* by adsorption emphasize the need for the prevention of pigment degradation during the preparation. To obtain chlorophyll *b*, the column must be washed exhaustively. Readsorption is always necessary, and if the amount of *a* is sufficiently low, it is possible to obtain the *a* and *b* bands as discrete zones. The third chromatogram should consist of a single *b* band and is a check on the complete removal of *a*.

The spectroscopic observations were made immediately after preparation of the final ether solution and before the weight data were available.

#### PRECISION AND RELIABILITY OF ABSORPTION SPECTRA

Under present operating conditions, the precision error of measurement has been found to be  $\pm 0.5\%$  for  $\log_{10} \frac{I_0}{I}$  values of 0.2–0.8, the working range given by HOGNESS, ZSCHEILE, and SIDWELL (4) for small values of  $\frac{\Delta I}{I_0}$ . It has also been demonstrated that total precision errors, including weight and volumetric technique, do not exceed  $\pm 0.6\%$ . The uncertainty between preparations is of course larger than this. As may be seen from the data, the maximum mean deviation from the average for all wave lengths listed is 1.83%; however, the average mean deviation is 1.22%. There is no significantly greater error in regions of high absorption than in regions of low absorption.

There are other factors which might influence absolute values obtained by this method. The spectral region isolated by the monochromator, as defined earlier (4), may influence the absorption coefficients obtained, depending on the wave length used and the nature of the absorption curve. The absorption value tends to be an average for all the wave lengths of radiation included in the spectral region employed. Values at narrow maxima or minima are therefore more influenced by variation of region isolated. The blue and red maxima of the chlorophylls are examples of this.

The absorption values for chlorophyll *a* solution have been studied as a function of spectral region isolated at the more critical wave lengths. Constant values were obtained under the following conditions:

4290 Å (maximum)	slits 0.03–0.25 mm.	spectral region 5–40 Å
4720 Å (minimum)	slits 0.04–0.5 mm.	spectral region 9–110 Å
6600 Å (maximum)	slits 0.02–0.06 mm.	spectral region 10–30 Å

In the case of chlorophyll *b*, constant values at the red maximum have been obtained for spectral regions isolated up to 38 Å. The slit widths employed in all the absorption measurements were within these ranges.

The radiation which reaches the solution has passed through much crystal quartz and has been reflected many times; it is therefore likely to be partially polarized. Absorption values for chlorophyll *a* at 6600 and 4290 Å were unchanged when a Polaroid disk was placed in the light path between the source and the condensing lens or directly before the absorption cell. Observations were made with the Polaroid disk at several rotational positions perpendicular to the light beam.

Determinations made on chlorophyll *a* with the following lines from a low pressure mercury arc agreed with those made on the same solution at the same wave lengths with a Mazda source: 4047, 4078, 4358, 4916, and 5461 Å.

Absorption values of chlorophyll *a* solutions at the 4290 Å maximum were observed when the following glass filters were placed in front of slit 1 of the monochromator: Jena BG7 (absorbs from 6400 Å to the red limit of photocell), Corning 385 (absorbs below 3700 Å), Corning 585 (absorbs from 5000 to 6900 Å), BG7 + 585, and BG7 + 585 + 385. At the 4720 Å minimum, Jena VG9 (absorbs from 6400 Å to the red limit and from 4400 Å to the ultraviolet limit of Mazda source), Corning 511 (absorbs from 5000 Å to the red limit), and both in series were used. No change was observed in the absorption coefficient in any of these experiments, demonstrating that error due to scattered radiation from the Mazda source is negligible under the conditions of these measurements.

The blue and red maxima of chlorophyll *a* preparations are found consistently at 4290 and 6600 Å and those of chlorophyll *b* at 4530 and 6425 Å, respectively. Determinations in these regions were made at 10-Å intervals. Other maxima and the minima are broad in comparison.

A straight line relationship between  $\log_{10} \frac{I_0}{I}$  value and concentration has been found at 4720, 5050, 4290, and 6600 Å for chlorophyll *a* in ether, for the concentration range employed.



## COMPARISON OF DATA

The curves in general are higher at the blue and red maxima than are those of other workers and lower at the minimum in the blue-green region (note particularly wave lengths 4720 and 5050 A for *a* and 4980 and 5200 A for *b*) as shown in figures 3 and 4. The values are equally accurate at the minima and maxima. The 4720 A minimum of *a* is particularly sensitive to the presence of carotenoid impurities. One % beta carotene would raise the absorption coefficient from 0.93 to approximately 3, an increase of 200%. This may explain high values at this wave length (see samples 1 and 6, table 1). Values at 4290 and 5050 A are much less sensitive to such impurities. Values at 4290, 5050, and 6600 A are sensitive to small amounts of pheophytin. It is apparent from figure 3 that most values from other laboratories at these wave lengths fall between our curves for component *a* and the corresponding pheophytin. The same is true for *b* at analogous wave lengths.

The curves show significant disagreement with those of MACKINNEY (7) and ZSCHEILE (13). Both workers employed the spectro-photoelectric method of measurement. Only maxima and minima and certain other pertinent points from ZSCHEILE's curves are plotted for comparison in figures 3 and 4. The conditions of preparation and measurement which are reported here are considerably improved over those under which ZSCHEILE worked (13). Only two concentrations were employed in these 1934 measurements, and some  $\log_{10} \frac{I_0}{I}$  values, particularly at the lower minima, were therefore not in the optimum range.

Inspection of the points plotted in figure 3 from ZSCHEILE (13) indicates that contamination with neither pheophytin nor colorless impurity alone could account for all the differences (see wave lengths 5050, 5275, and 6300 A). High pheophytin content is not indicated, but colorless impurities were probably present because the adsorbent was removed merely by filtration through filter paper. It is interesting to note that ZSCHEILE's values at 4290 and 6600 A are both 75% of those reported here. Some values, such as those at 5050, 6600, and 4290 A, might be considered to result from a combination of these two sources of error.  $R_a$  values as high as that of ZSCHEILE have not been obtained with the two brands of talc available here, when solutions were dried. These and other data indicate that samples of talc differ considerably in the amount of pigment decomposition resulting when chlorophyll is adsorbed on them. The  $R_a$  values differ by a factor as high as 3 when sucrose and talc adsorbents are compared (dried preparations).

Similar considerations apply in the case of *b*; however, larger differences are involved at certain wave lengths. In 1936 ZSCHEILE (14) prepared component *b*

by extending his earlier method (13) to include two successive adsorptions on sucrose. The last traces of *a* were observed as a thin layer separated from the *b* layer on the last column. Sucrose was removed by washing with water and evaporating the solution to dryness. Maximum specific absorption coefficients of 147 and 49.5 were obtained at the blue and red maxima, respectively. These values approach those reported by MACKINNEY (7). His reported values at the blue and red maxima for both components are somewhat lower than ours, and his values at the low minima are higher. However, he does not claim great accuracy for these latter values.

The curves of chlorophyll from Sandoz, Ltd., indicate the magnitude of error that may arise from the use of such preparations as spectroscopic and colorimetric standards. These samples, in common with many others, show high absorption in the region near 5000 Å. The present data indicate why MEYER (8) obtained increased values near 5300 Å after drying his "native chlorophyll" and allowing it to stand. Drying rendered his standard chlorophyll unreliable. We found that when a chlorophyll *a* solution in ether was dried by nitrogen which was purified by passage over hot copper, the value of  $R_a$  decreased as much as when dried in air. MEYER also found that increase of values near 5300 Å was independent of the presence of oxygen.

HAGENBACH *et al.* (2) used chlorophyll from Sandoz, Ltd., and—after repurification by adsorption—measured the spectra with König-Martens and Hilger spectro-photometers. Their value of 231 for chlorophyll *b* at 4499 Å is too high to plot in figure 4. They obtained equal values for *a* and *b* at the red maxima. We offer no explanation for the unusually high values of these workers.

RUDOLPH (9) prepared *a* and *b* by the methods of WILLSTÄTTER and STOLL and of SCHERTZ and observed the spectra with a König-Martens spectro-photometer. His curves do not cross in the green and red regions. They are so low and lacking in detail as to indicate gross impurity, degradation, or both.

HEIERLE (3) employed chlorophyll supplied by STOLL and used a Zeiss grating spectrograph with a Baly absorption cell. His values are in general low in the red and blue regions and high in the green. His curve for *b* is particularly poor in the red region, since the shelf near 6600 Å indicates contamination with either component *a* or a pheophytin.

We have observed that the initial degradation by drying of chlorophyll solutions causes the absorption curve to fall intermediate between the curves for the pure component and its corresponding pheophytin, at most wave lengths. Very large amounts of degradation, as caused by exposure to light at room temperature, cause decomposition of pheophytin also, and thus produce a general flattening of the entire curve, with attendant disappearance of detail. This complex course of

events makes impossible at the present time the accurate treatment of data from various laboratories, at least so far as definite statements of detailed causes for differences are concerned.

MACKINNEY (6) has stressed the importance of obtaining the chlorophyll components in a pure solid form. On the basis of results presented here, it does not seem probable that the components of chlorophyll, in a stable and pure form, will be generally available for use as spectroscopic standards.

It is hoped that careful observations of certain precautions discussed here may contribute to an early agreement among workers in this field of pigment research.

### Summary

1. An improved method for preparation of chlorophyll component solutions with reproducible spectroscopic properties is presented. The method is rapid and simple; sucrose is used, both for filtration of precipitated pigment and for separation of components by adsorption.

2. The absorption spectrum in ether solution has been shown to be very sensitive to previous treatment of the solution. It is emphasized that drying must be avoided and that spectroscopic observations must be made very soon after purification.

3. The spectra of acidified chlorophyll solutions are presented, and their relation to ordinary degradation effects is discussed. Some comparisons with reported values are made.

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# OÖGENESIS AND FERTILIZATION IN *PINUS LAMBERTIANA* AND *P. MONOPHYLLA*

ARTHUR W. HAUPT

(WITH TWENTY-SIX FIGURES)

## Introduction

An investigation of the cytological features associated with the maturation and fertilization of the egg was undertaken in two species of pines occurring in southern California. Both produce large ovules that may be easily removed from their cones and dissected. The cones of *Pinus lambertiana* reach a greater length than those of any other species, but being borne on tall trees, are difficult to obtain. Although much smaller, the cones of *P. monophylla* occur in great abundance on trees of low stature having branches close to the ground.

## Material and methods

Nearly all the material of *Pinus lambertiana* was collected in the San Bernardino Mountains north of Redlands, California, at elevations ranging from 5700 to 7200 feet. Collections were made daily from June 21 to June 24, 1935. As might be expected, cones from a lower altitude were in a later stage of development than those from a higher altitude. On June 21, material gathered at 5700 feet showed many cases of fertilization, while at 7200 feet the archegonia revealed an undivided central cell, or occasionally one in process of division. On June 24, fertilization stages were found only in material obtained at 6400 feet, that collected at lower altitudes being too old and at higher altitudes too young. At any given elevation, about 5 days were found to elapse between the cutting off of the ventral canal cell and fertilization.

Material of *Pinus monophylla* was obtained in Cuddy Canyon, 2-4 miles west of the point where Los Angeles, Kern, and Ventura counties meet. It came from various elevations between 4300 and 5200 feet. Material collected on June 8 at 4300 feet included an abundance of fertilization and young proembryonal stages; that gathered at higher elevations was younger. On June 15, material growing at 4300 feet had reached a proembryonal stage, with walls formed and suspensors elongating; at 5200 feet the ovules were much younger, only a few having reached the fertilization stage. Collections made on June 1, 1936, and May 31, 1937, at 4300 feet showed the nucleus of the central cell just ready to divide in some ovules, in others just having divided. Another collection at the same elevation on June 4, 1937, yielded many cases of fertilization.

In both species, the interval between pollination and fertilization is almost exactly one year. In *P. lambertiana* the staminate cones opened a day or two after ovules in fertilization stages had been collected from the same trees. In *P. monophylla* the staminate cones at the time of fertilization were just beginning to shed their pollen and the young ovulate cones were just emerging from their buds. In the large cones of *P. lambertiana* the ovules near the apex might be in the mature-egg stage, fertilization not yet having occurred, and those near the base in an early proembryonal stage, with free nuclei at the center or base of the egg. In the small cones of *P. monophylla*, on the other hand, the ovules are more nearly in the same stage of development throughout. At any particular time, however, some variation occurs among cones taken from the same tree, and still more among cones taken from different trees growing at the same elevation.

Upon removal from the trees, the cones were wrapped in waxed paper and placed in air-tight containers. Two or 3 hours later they were brought to the laboratory for dissection, the ovules meanwhile having lost none of their turgidity. Because of the large size of the ovules, the female gametophyte could easily be removed, a procedure greatly facilitating penetration of the fixing fluid. Except in early stages, it was usually possible to retain the nucellus in place at the apical end of the female gametophyte.

The material was satisfactorily fixed in Navashin's fluid, prepared according to the formula given by CHAMBERLAIN (4). Shrinkage of the egg, which nearly always follows the use of ordinary methods, was avoided by leaving the material in the fixing fluid for at least 3-4 days, washing thoroughly, and carrying it through a very closely graded series, first of ethyl alcohol and then of xylol. The sections were cut 12  $\mu$  thick and were stained in iron-alum haematoxylin.

### Historical résumé

The literature dealing with the cytological aspects of fertilization in conifers is not extensive, the principal papers being those of BLACKMAN (2) on *Pinus sylvestris*, FERGUSON (6, 7) on *P. strobus*, HUTCHINSON (8) on *Abies balsamea*, and BEAL (1) on *P. banksiana*. Oögenesis was studied by both BLACKMAN and FERGUSON, while CHAMBERLAIN (3) on *P. laricio* was concerned mainly with oögenesis. Fertilization has been described, in considerably less detail and incidental to other features of the life history, by MURRILL (12) in *Tsuga canadensis*; LAWSON (9, 10, 11) in *Sequoia sempervirens*, *Cryptomeria japonica*, and *Cephalotaxus drupacea*; NORÉN (14) in *Juniperus communis*; and NICHOLS (13) in *J. communis* var. *depressa*.

The most recent study of fertilization in conifers, that of BEAL (1), deals with the behavior of the chromosomes following the conjugation of the male and female nuclei. It confirms in all essential respects and adds details to the earlier investiga-

tions of BLACKMAN, FERGUSON, and others, who found that in the first embryonal mitosis the paternal and maternal chromosomes split longitudinally, as in any other vegetative mitosis, and that the number of chromosomes in the fertilized egg is diploid.

BEAL's results are in sharp disagreement with those of HUTCHINSON (8), who reported that the members of the paternal set of chromosomes pair with those of the maternal set, that each chromosome undergoes transverse rather than longitudinal segmentation, and that the segments thus formed separate, half going to each pole of the spindle. After pairing, but before separation, the chromosomes were seen to twist about each other and become looped, a behavior suggestive of that occurring in meiosis. HUTCHINSON reported that the number of pairs of chromosomes is haploid; BEAL found no pairing and no transverse segmentation, but a diploid complement of chromosomes, each of which is split longitudinally.

### Investigation

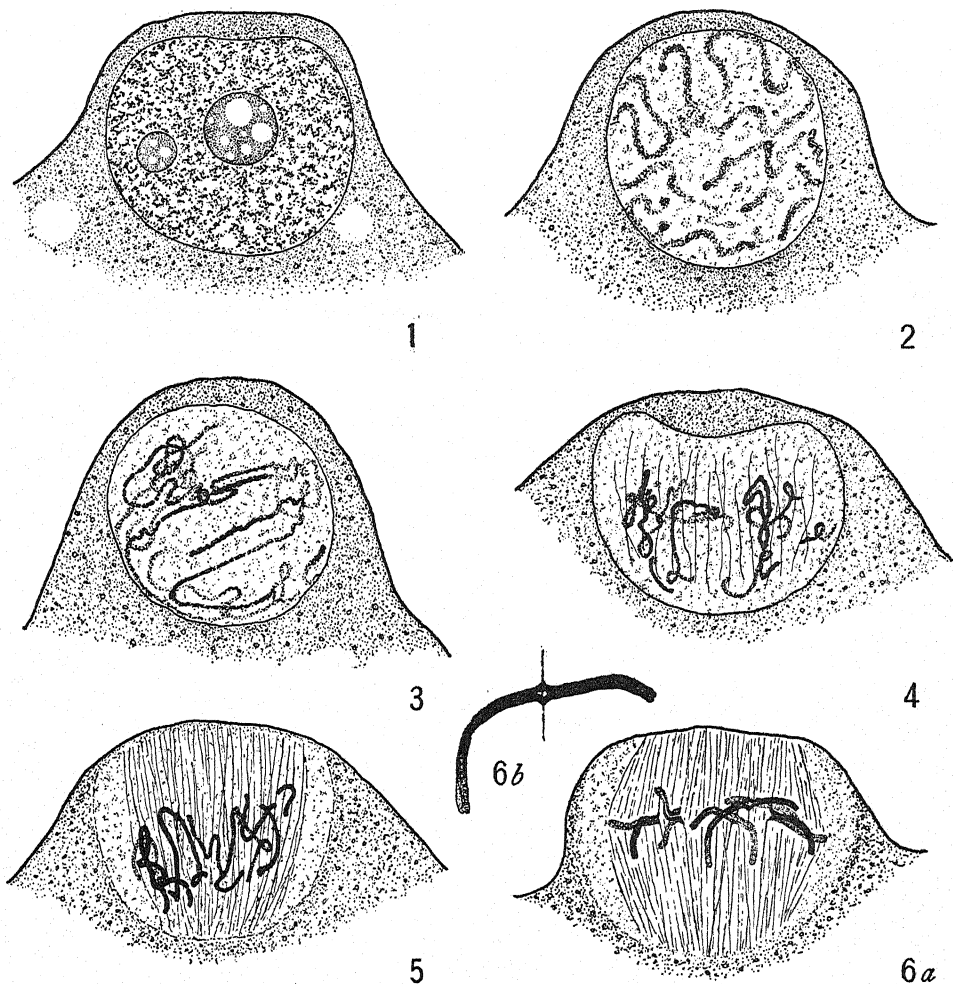
Except where otherwise noted, the following statements apply to both species of *Pinus* studied.

### CENTRAL CELL

The cytoplasm of the central cell consists of a finely granular ground substance, without fibrous elements, containing numerous vacuoles confined chiefly to the central region and ranging in diameter from 5 to 100  $\mu$  (occasionally up to 200  $\mu$ ). Deeply staining food particles are distributed in great abundance throughout the cytoplasm. At first they are very small and seem to form an irregular reticulum, but later increase in diameter up to 2  $\mu$  and appear separate and distinct. The "proteid vacuoles" of earlier writers are organized within the central cell as it matures. They consist of a distinct membrane inclosing food particles that have the same appearance as those occurring in the general cytoplasm. They attain a diameter of 30-40  $\mu$ . At first these proteid bodies are restricted to the bottom and sides of the cell, where the cytoplasm is less vacuolated, but as the vacuoles in the central region decrease in size and number, they become more abundant and more evenly distributed.

The nucleus is situated at the extreme apical end of the central cell, just below the single tier of neck cells (fig. 1). It is most commonly ellipsoidal and flattened or concave above, varying in its greatest diameter from 45 to 55  $\mu$ . A small amount of cytoplasm, sometimes containing several small vacuoles, is present above the nucleus. The nuclear material is more coarsely granular than the cytoplasm, but stains almost as lightly. Apparently it consists of a single substance forming an irregular network in which no linin can be distinguished. The nucleus generally contains several nucleoli, the largest of which may be 15  $\mu$  in diameter. Commonly two nucleoli are present, one being about twice as large as the other.

They stain somewhat darker than the nuclear network and are conspicuously vacuolated. FERGUSON (6, 7) states that the largest nucleolus is invariably central in position, but this is not true of either of the present species.



FIGS. 1-6.—Early stages in division of central cell nucleus of *P. monophylla*: fig. 1, resting nucleus; fig. 2, early stage in differentiation of chromatic threads; fig. 3, later stage, chromatin confined to periphery of nucleus; fig. 4, chromosomes and spindle fibers; fig. 5, disappearance of nuclear membrane; fig. 6a, equatorial-plate stage; fig. 6b, single chromosome showing longitudinal splitting and fiber-attachment points. Fig. 6b,  $\times 1250$ ; others,  $\times 625$ . Figs. 3, 5, and 6 represent one section from a series of three; figs. 2 and 4, one section from a series of four.

Some preparations show a slight withdrawal of the central cell from the neck cells, while others do not. Often torn strands of cytoplasm extend into the in-



tervening space, indicating that the withdrawal is due to shrinkage arising from imperfect methods of preparation. For the same reason the central cell occasionally is pulled away from the archegonial jacket. By the time the division of its nucleus takes place, the central cell has reached an average diameter and length of approximately 400 and 700  $\mu$ , respectively, but sometimes a length of 1000  $\mu$  may be attained, especially in *P. monophylla*. As a rule the vacuoles have disappeared from the cytoplasm, but often a few persist in the apical region. FERGUSON (6, 7) finds only a few proteid bodies at this stage, but in my material they are abundant even somewhat earlier.

The number of archegonia in a single ovule is somewhat variable, but is most commonly five in *P. lambertiana* and generally three in *P. monophylla*.

#### DIVISION OF CENTRAL CELL

As previously stated, the resting nucleus seems to contain a single granular material, somewhat coarser than the cytoplasm, and forming an interrupted reticulum. Preparatory to nuclear division, some of this material becomes aggregated to form loose clumps that show no regularity in form or distribution. By a further consolidation of their particles, these irregular clumps soon attain a more definite shape, and their appearance now suggests that they represent differentiated strands of chromatin (fig. 2). As it loses its granular character and becomes more compact, the chromatin assumes a deeper stain. Meanwhile the nucleoli disappear. Soon the chromatin becomes drawn out into long slender threads sharply differentiated from the surrounding granular material of the nucleus (fig. 3). They do not appear to form a continuous spireme. These threads have a monilliform appearance and portions tend to be highly convoluted. In some preparations the chromatin at this stage lies in the peripheral portion of the nucleus.

A later stage in the division of the central cell nucleus reveals an increased consolidation of its chromatin to form definite chromosomes and the appearance of spindle fibers (fig. 4). The chromosomes are very long, slender, and coiled. A monilliform structure is no longer apparent. At first the chromosomes are distributed throughout the nucleus, and because many are cut by the microtome knife, it was not possible to determine their number. At this stage the chromosomes are so slender that it could not be ascertained whether they are longitudinally split, although there is some evidence that such is the case. The spindle fibers make their appearance entirely within the nuclear membrane. They are relatively few in number and extend vertically in an irregular manner.

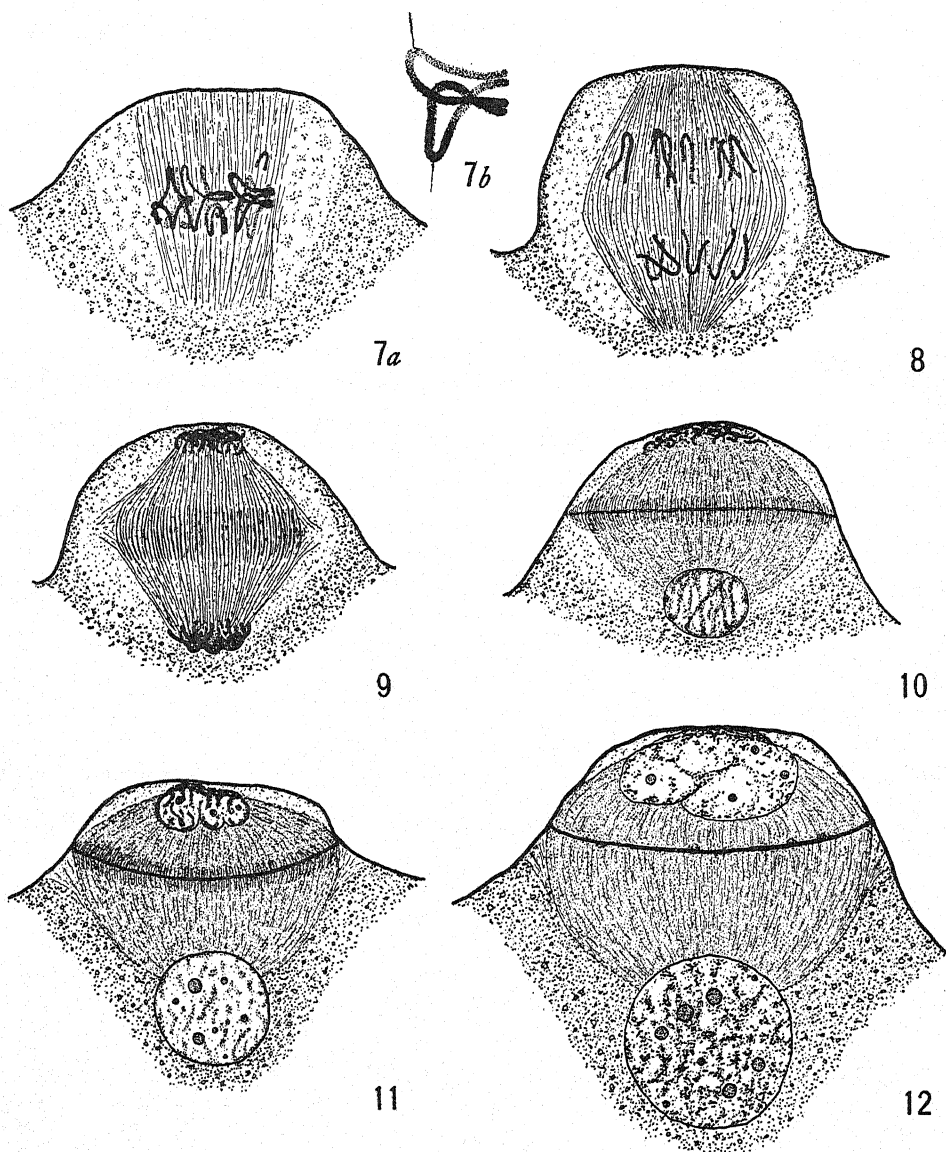
Presently there is formed a well-defined, intranuclear, multipolar, diarch spindle extending throughout the entire length of the nucleus (fig. 5). It is as broad above as at the equator (30–35  $\mu$ ), or nearly so, but is usually somewhat narrower below. The chromosomes are now grouped in the central portion of the spindle and are

spreading out in a horizontal plane. They are still long, slender, and much curved, the longest being 500–550  $\mu$ . Often longitudinal splitting can be clearly seen, and sometimes it is possible to discern fiber-attachment points. Lying outside the spindle and reaching to the nuclear membrane, which is now breaking down, is the lightly staining granular material not previously involved in the formation of chromatin. During this stage and subsequent ones, the nucleus generally lies so close to the apical end of the central cell that little or no cytoplasm is present above it.

Many metaphases were studied. The nuclear membrane has now almost or entirely disappeared. The spindle remains broad at the poles, granular material derived from the resting nucleus surrounding it on all sides. Often the spindle is as broad at each pole as at the equator, but usually it is slightly narrower below. BLACKMAN (2) states that the spindle is bluntly pointed at both ends; FERGUSON (6, 7) finds that the lower end forms a single pole while the upper remains multipolar. By this time the chromosomes have become much shorter, as well as thicker, denser, and more or less U-shaped (fig. 6*a*). They are arranged somewhat regularly on the equatorial plate, the ends tending to point outward. The fiber-attachment points are now conspicuous, being situated at or near the middle of the chromosomes (fig. 6*b*). In most cases the presence of 12 chromosomes can be determined with certainty. Longitudinal splitting can be more readily observed as the chromosomes continue to shorten and thicken. At the equatorial-plate stage the chromosomes are much twisted, particularly near their ends. This is very apparent somewhat later, when the halves of each split chromosome separate (fig. 7*a, b*).

During the anaphase the chromosomes are V-shaped or narrowly U-shaped (fig. 8). In both species, 12 chromosomes going to each pole can be easily counted. Although the nuclear membrane has disappeared, the granular material persisting from the resting stage still surrounds the spindle and usually can be distinguished from the cytoplasm. As the chromosomes approach the poles, the fiber-attachment points are somewhat less evident, but still may be recognized. The spindle is now elliptical in outline and narrower at the poles. The lower group of chromosomes becomes more compact, while the upper one spreads out horizontally.

As soon as the chromosomes have reached the poles, a slight thickening of the mantle fibers across the equator of the spindle is the first indication of cell-plate formation (fig. 9). In a slightly later stage the spindle has broadened at the equator by the appearance of additional fibers, and the cell plate has formed across it (fig. 10). The lower group of chromosomes now forms a compact, spherical, lightly staining mass inclosed by a thin nuclear membrane, while the upper group, which stains more intensely, usually becomes flattened out against the cell wall. By this time the persistent granular material of the resting nucleus is barely discernible,



FIGS. 7-12.—Late stages in division of central cell nucleus of *P. monophylla*: fig. 7a, late metaphase; fig. 7b, single pair of daughter chromosomes nearly separated; fig. 8, anaphase; fig. 9, early telophase; figs. 10-12, later telophases showing cell wall formation. Fig. 7b,  $\times 1250$ ; others,  $\times 625$ . Fig. 7 represents one section from a series of two; fig. 8, one section from a series of three.

having largely merged with the cytoplasm. The egg nucleus is about  $15-20\ \mu$  in diameter.

The completion of the outward growth of the cell wall that cuts off the ventral canal cell from the egg is shown in figure 11. This wall has now become thickened and slightly concave upward. The egg nucleus,  $25-30\ \mu$  in diameter, is spherical or sometimes flattened above. The chromosomes are no longer distinct, but at least a portion of their original substance can be identified as faint, narrow, more or less vertical bands. The coarsely granular material has made its appearance in the nucleus and seems to have arisen from disintegration of the chromosomes, at least in part. Usually a number of small vacuolated nucleoli are present also, but these may be wholly absent. In figures 11 and 12 the ventral canal nucleus is flattened and more or less lobed below but shows much the same structure as the egg nucleus. In many cases, however, the ventral canal nucleus does not reach the resting condition, its chromatin remaining as an indistinct mass that soon disorganizes without forming a nuclear membrane. The ventral canal cell persists as a small, lenticular, deeply staining mass lying between the egg and the neck cells.

In *P. sylvestris*, BLACKMAN (2) describes the nucleus of the ventral canal cell and that of the egg as exactly similar, stating that the former "surrounds itself with a membrane and goes through the earlier stages, at least, of the normal process of return to the resting condition . . . but soon after its formation loses its membrane and appears as a number of irregular rods and clumps." FERGUSON (6, 7), on the contrary, finds that the nucleus of the ventral canal cell, although highly variable, rarely approximates the egg nucleus in size and form, and that there are probably instances in which no nuclear membrane appears. It undergoes early disintegration, hardly ever presenting a normal appearance.

#### MATURE EGG

As the egg nucleus migrates to a central position it becomes ellipsoidal and attains an average diameter and length of approximately  $150$  and  $200\ \mu$ , respectively. While it is still under  $100\ \mu$  in diameter, usually some evidence of chromosome structure may be faintly seen, but this soon disappears. When its maximum size has been reached, the egg nucleus consists of a coarsely granular substance forming an interrupted reticulum and showing no evidence of linin. It exhibits a structure very similar to that of the resting nucleus of the central cell. Sometimes no nucleoli can be recognized, but generally a variable number are present. As a rule there are several small nucleoli, each  $5-8\ \mu$  in diameter and with a central vacuole. In addition there is usually a single large nucleolus,  $20-30\ \mu$  in diameter, which contains so many vacuoles that it presents a frothy appearance. Sometimes a great many small nucleoli are seen, in one instance in *P. lambertiana* as many as 130

having been counted in the whole nucleus. The nucleoli are in groups, but the groups are not confined to any particular part of the nucleus.

BLACKMAN (2) considers that the young egg nucleus "consists of a small number of chromatin bands and a few round nucleoli." As it enlarges and moves downward, it becomes filled with a peculiar substance, absorbed from the cytoplasm, that earlier had been designated as metaplastm by STRASBURGER (15), who claimed that it entirely obscures the chromatin. BLACKMAN found that at first the metaplastm is granular and clearly distinct from the chromatin, but later the two are indistinguishable, the whole female nucleus being filled with a reticulum that, although not chromatin, "stains uniformly in a chromatin-like manner." In addition, there is present either a large hollow nucleolus or a number of small nucleoli.

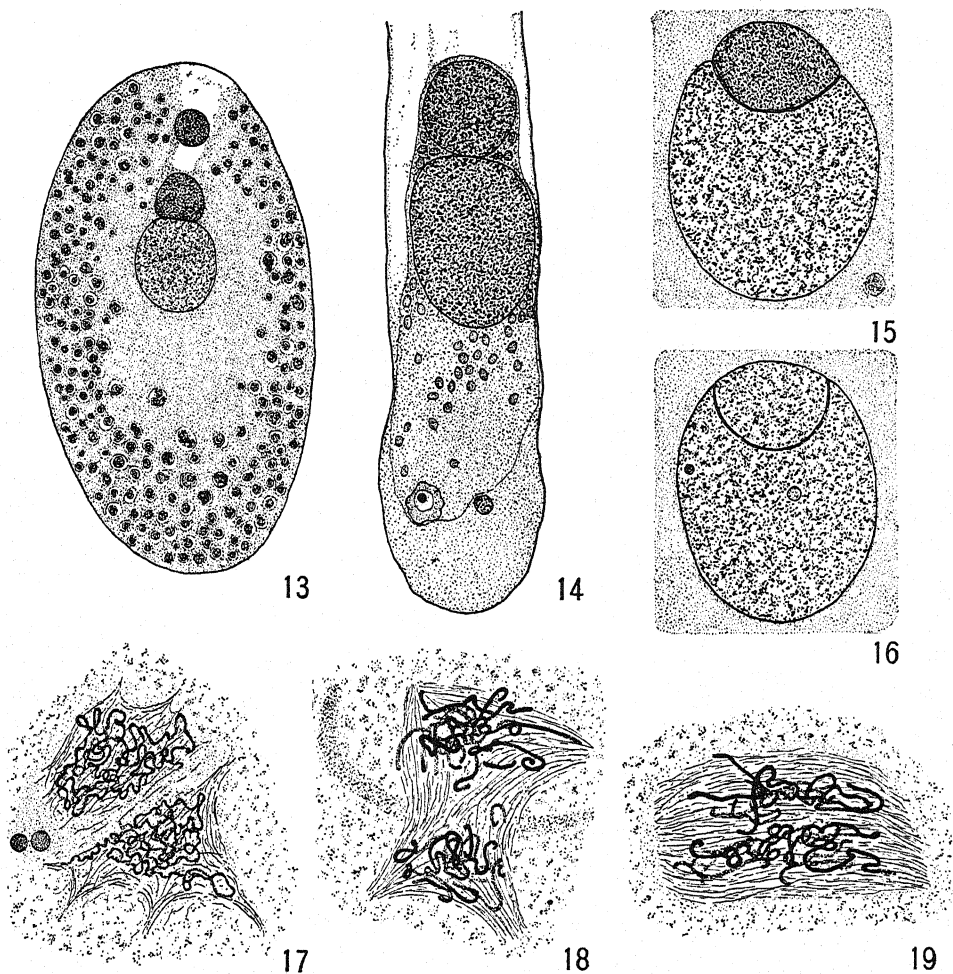
CHAMBERLAIN (3) states that, in the development of the egg nucleus the chromatin takes the form of numerous small nucleoli arranged upon a linin network. This resolves itself into a granular substance, leaving the nucleoli irregularly scattered. He finds that some of the nucleoli contribute to the granular substance, while others come together and give rise to elongated chromatin masses, at which time a nuclear reticulum again appears. CHAMBERLAIN states that "the term metaplastm is not used in the present paper because the network is regarded merely as a somewhat peculiar linin network."

FERGUSON (6, 7) likewise does not recognize the presence of a metaplastmic substance in the egg nucleus. She describes an open interrupted reticulum on which are arranged irregular granules. The linin, always abundant, may form heavy hyaline cords or less conspicuous strands. She finds chromatin to "exist either in the form of irregular granules of varying sizes or apparently dissolved in the linin," but did not observe such a resolving of the chromatin into nucleoli as described by CHAMBERLAIN. FERGUSON reports the occasional occurrence of nucleoli in *P. austriaca* during early developmental stages, but finds them rarely present in *P. strobilus* at this time. Later she says, "small nucleolus-like bodies, containing a single vacuole, appear in connection with the nuclear net; and at the same time a slightly larger nucleolus is observed in the lower part of the nucleus, usually in connection with its membrane. As the nucleus continues to grow, this nucleolus also increases in size, gradually becoming large and very vacuolate." FERGUSON finds that there may be several smaller nucleoli, a great many, or none.

After the cutting off of the ventral canal cell, the egg does not increase in size. Its cytoplasm becomes filled with the deeply staining food granules that made their appearance earlier, while the vacuoles are nearly or entirely gone (fig. 13). FERGUSON has expressed the opinion that proteid bodies arise within the nucleoli of the egg and sheath cells. The present study has afforded no evidence that this is true.

## POLLEN TUBE

As the pollen tube approaches the archegonia, its contents are massed at the lower end (fig. 14). Here may be distinguished, surrounded by dense cytoplasm containing numerous starch grains, the small tube nucleus and stalk cell, as well as



FIGS. 13-19.—Conjugation of sexual nuclei and stages immediately following: fig. 13, two male nuclei within egg, the larger, anterior one in contact with female nucleus,  $\times 95$ ; fig. 14, lower end of pollen tube just before penetration of egg, showing small tube nucleus, stalk cell, and two large male nuclei,  $\times 250$ ; figs. 15, 16, penetration into female nucleus,  $\times 185$ ; fig. 17, male and female chromatin in separate groups, each surrounded by spindle fibers,  $\times 625$ ; fig. 18, later stage, with two groups of chromosomes on common polyarch spindle,  $\times 625$ ; fig. 19, still later stage, with chromosomes still in two groups but spindle becoming diarch,  $\times 625$ . Figs. 13, 14, and 17 are of *P. lambertiana*; figs. 15, 18, and 19 are of *P. monophylla*.

the two large male nuclei of unequal size. The tube nucleus contains many deeply staining chromatin granules. The male nuclei are inclosed by a common mass of cytoplasm derived from the body cell, which is separated from the general cytoplasm of the pollen tube by a delicate membrane. Each of the male nuclei consists entirely of the same kind of coarsely granular material as that which fills the egg nucleus, but neither at this time nor subsequently are any nucleoli to be seen. The male nuclei lie in contact with each other, the smaller one being behind. In figure 14 the smaller male nucleus measures  $50\ \mu$  in diameter, the larger one  $70 \times 90\ \mu$ . FERGUSON (5, 7), studying several species of pines, finds the contents of the pollen tube essentially the same as just described, except that the male nuclei, having a dense reticulum, contain no special metaplasmic substance.

#### CONJUGATION OF SEXUAL NUCLEI

Before the contents of the pollen tube enter the archegonium, a large saclike cavity appears in the upper portion of the egg and persists during its subsequent development, a feature noted by earlier workers. Most of the male cytoplasm enters the egg but does not surround the anterior, functional, male nucleus as it approaches the female. The second male nucleus, and generally the tube nucleus aslo, can be identified in the upper part of the egg, but as a rule the stalk cell does not persist.

When the functional male nucleus reaches the egg nucleus, the two become flattened along the contact surface, while a thin layer of cytoplasm lies between them (fig. 13). As previously pointed out by BLACKMAN (2) and FERGUSON (6, 7), the male nucleus sinks into the female nucleus, forming an invagination, the nuclear membranes meanwhile remaining intact (fig. 15). This continues until the outer surface of the male nucleus reaches the same level as that of the female (fig. 16). Occasionally a few small masses of cytoplasm are caught between the two nuclei where their membranes are in contact. Generally at this time the egg contains no nucleoli, but sometimes several small ones may be present.

No change takes place in the structure of the functional male nucleus as it approaches the egg nucleus, and when the two first come into contact the former is always denser than the latter. As the male nucleus sinks into the female nucleus it becomes less dense, until finally the two are of equal density (figs. 15, 16). The two male nuclei do not increase in size as they enter the egg, nor do they enlarge subsequently. They are usually ellipsoidal or ovoid, but either or both may be spherical.

#### FIRST EMBRYONAL MITOSIS

As the portions of the membranes of the fusing nuclei that are in contact slowly break down, two groups of chromosomes are differentiated in the immediate vicinity, one from the male nuclear material and the other from the female. In

each group the chromatin takes the form of attenuated, very slender, convoluted strands; at the same time two groups of spindle fibers make their appearance (fig. 17). Although somewhat irregularly arranged, the fibers in each group tend to form a multipolar polyarch spindle. These spindles, like the male and female chromatin groups, lie close together, but when first apparent are separate. They soon converge to form a common spindle, upon which the two groups of chromatin lie (fig. 18).

The slender chromatin threads become shorter and thicker, assuming the form of chromosomes. These are long, slender, and curved, sooner or later losing the moniliform appearance characteristic of earlier stages. As the two groups of chromosomes come together, the common multipolar spindle on which they lie gradually becomes diarch (fig. 19). Even now the paternal and maternal groups are recognizable, but soon lose their identity. The female chromatin occupies a relatively small part of the egg nucleus, lying close to the chromatin contributed by the male nucleus. The rest of the egg nucleus contains the same kind of coarsely granular material found before the chromatin became differentiated (fig. 20).

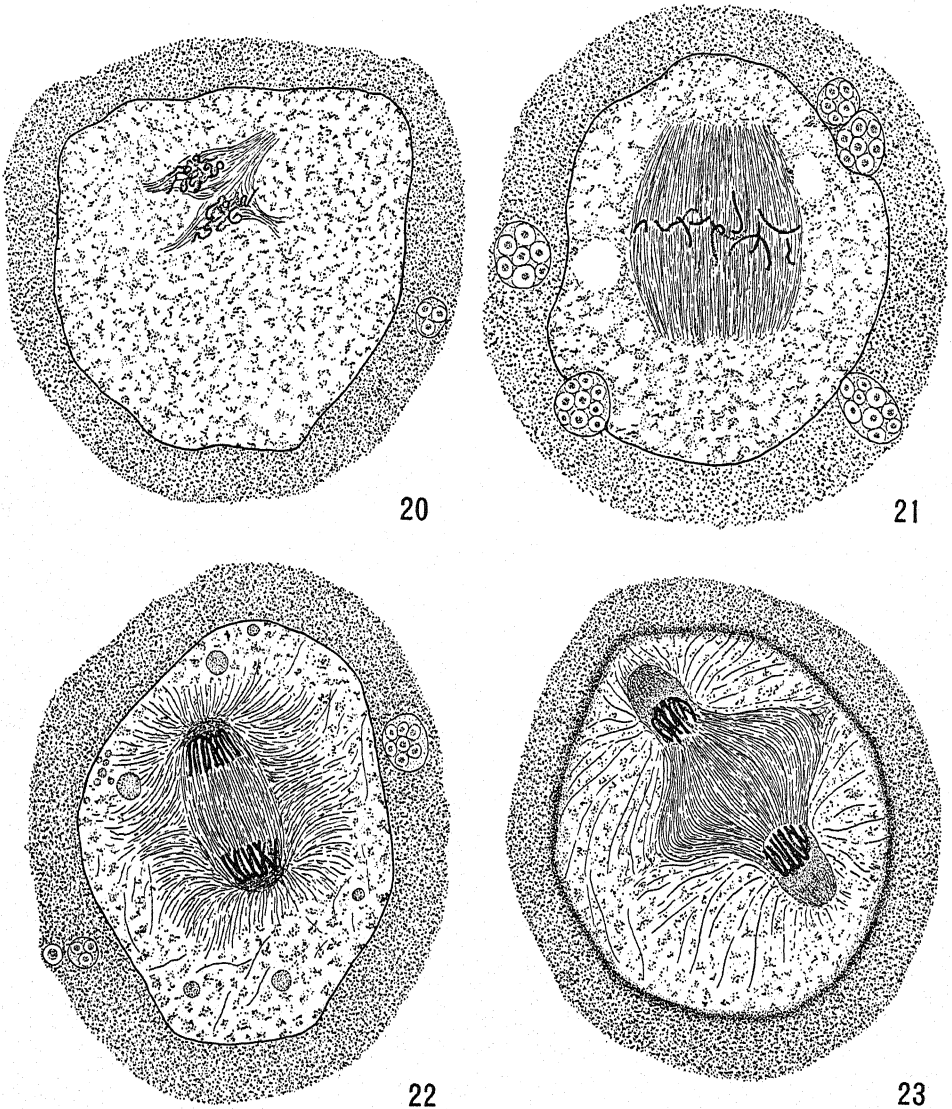
FERGUSON (6, 7) finds in each of the two sexual nuclei, after they have come into contact, that the chromatin condenses to form a spireme "studded with irregular granules." This soon becomes coiled and irregularly moniliform, leaving the remainder of the nucleus filled with a granular reticulum composed of linin. Delicate threads appear near the spiremes, later forming an irregular, multipolar, polyarch spindle that often consists of two parts. By this time the chromatic bands have become homogeneous. Finally the spindle becomes multipolar and diarch, "and the achromatic substance not used in spindle-formation has been gradually resolved . . . into a granular, or finely reticulated structure, which later merges into the general cytoplasm of the egg."

After the paternal and maternal chromosomes have become arranged at the equator of the spindle, they are indistinguishable from each other (fig. 21). The spindle itself, which remains multipolar and diarch, is very broad. It is usually 50-70  $\mu$  in diameter, but sometimes may reach a width (in *P. monophylla*) of 120  $\mu$ , being then several times broader than long. At first the chromosomes are attenuate and very slender, often showing clearly the moniliform structure characteristic of earlier stages. Soon they become shorter, thicker, and somewhat U-shaped (fig. 24a-f). The fiber-attachment points, which are median, are always clearly discernible. Longitudinal splitting of the chromosomes is not evident at first, but soon becomes so, particularly where the spindle fibers are attached.

When longitudinal sections 12  $\mu$  in thickness are cut through the mitotic figure, the chromosomes are distributed over a series of at least five or six sections, with the result that many of them are cut into several pieces. But, as BEAL (1) has shown, it is possible to estimate the number of whole chromosomes present in the



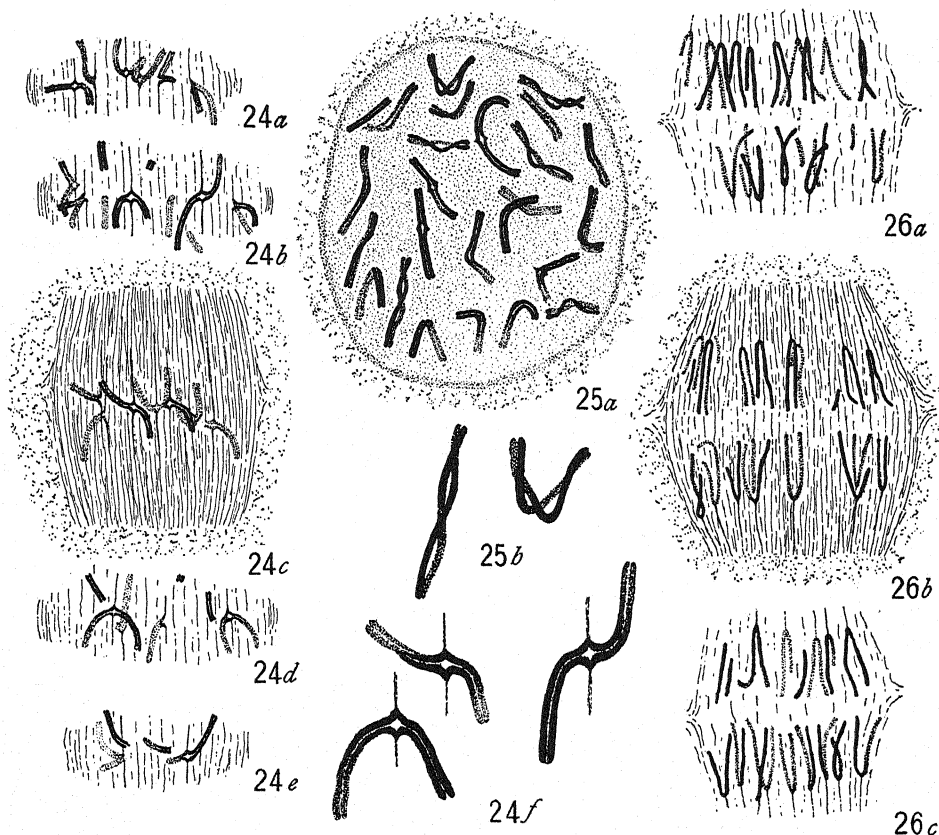
figure before it was sectioned by counting the pieces with fiber-attachment points. Using this method, BEAL found in *P. banksiana* approximately 24 chromosomes at



FIGS. 20-23.—First embryonic mitosis in *P. monophylla*,  $\times 360$ : fig. 20, egg nucleus with male and female chromatin differentiated; fig. 21, metaphase; fig. 22, anaphase; fig. 23, telophase. Fig. 21 represents one section from series of six; figs. 22 and 23, one section from series of two.

the metaphase of the first embryonic mitosis. In the many preparations of this stage available for the present investigation, in both species, the presence of 24

chromosomes could often be established with considerable accuracy (fig. 24a-e). In no case was there evidence of a haploid number of paired chromosomes, as described by HUTCHINSON (8) in *Abies balsamea*.



FIGS. 24-26.—Chromosome behavior during later stages of first embryonal mitosis: fig. 24a-e, consecutive longitudinal sections through entire mitotic figure of *P. lambertiana* at metaphase, showing diploid complement of 24 chromosomes, the number in each section with fiber-attachment points being 6, 5, 7, 4, 2; fig. 24f, 3 chromosomes from same, showing longitudinal splitting; fig. 25a, transection through mitotic figure of *P. monophylla* in late metaphase, showing polar view of diploid complement of 24 chromosomes; fig. 25b, two pairs of daughter chromosomes from same, showing twisting; fig. 26a-c, consecutive sections through entire mitotic figure of *P. lambertiana* at anaphase, showing 24 daughter chromosomes passing to each pole. Figs. 24f and 25b,  $\times 1250$ ; others,  $\times 625$ .

FERGUSON (6, 7) states that the two groups of chromosomes remain distinct until oriented on the equatorial plate. At this stage she demonstrated in *P. strobus* the presence of 24 chromosomes in a single cross section. She first observed longitudinal splitting at the metaphase, stating that "each chromatic element divides at the point where the spindle-fibers are attached, forming a small diamond-

shaped opening." BEAL did not find a polar view of the equatorial-plate stage, but of several seen in the course of the present investigation, that of *P. monophylla* (fig. 25*a*) includes the full diploid complement of 24 chromosomes. Each chromosome is longitudinally split and the segments show a pronounced tendency to twist about each other (fig. 25*b*). This twisting becomes apparent in late metaphase stages, both in the first embryonal mitosis and in the mitosis concerned with the cutting off of the ventral canal cell.

Because of the median position of the fiber-attachment points, the daughter chromosomes—upon separation—become V-shaped or narrowly U-shaped. It is not difficult to determine, during the anaphase, that 24 chromosomes pass to each pole (fig. 26*a-c*). At this time the fibers on the outside of the spindle tend to pass outward into the nucleus without extending directly from pole to pole, and this tendency becomes accentuated during the telophase, when a display of achromatic structures is very evident (figs. 22, 23). The telophase is also characterized by a marked broadening of the spindle and the appearance of achromatic material in the form of polar caps. At this time the nuclear membrane of the egg breaks down, allowing the granular metaplasmic material of the nucleus to merge with the surrounding cytoplasm (fig. 23).

As observed by BLACKMAN (2), the daughter nuclei resulting from the first embryonal division are at first very small, but later take up metaplasmic substance and often reach one-third the size of the egg nucleus.

#### SECOND EMBRYONAL MITOSIS

In the second embryonal division BEAL (1) states that the chromosomes have approximately the same general form and size as in corresponding stages of the first division; moreover, "the manner of separation at the fiber-attachment points and the indications of longitudinal splitting are practically identical in the two divisions." The present study fully confirms these statements.

#### Discussion

The presence in the mature egg nucleus of a metaplasmic substance that is absorbed from the cytoplasm and entirely obscures the chromatin, as claimed by STRASBURGER (15) and BLACKMAN (2), is confirmed by the present investigation. The same substance is present in the male nuclei and in the nucleus of the central cell. No evidence was found supporting the contention of CHAMBERLAIN (3) and FERGUSON (6, 7) that the nuclear reticulum consists primarily of linin.

The behavior of the chromosomes in the first embryonal mitosis closely parallels that in the division of the central cell nucleus, except that the diploid rather than the haploid number is involved. In both cases the chromosomes arise from

the resting nucleus in the same way, undergo longitudinal splitting, and exhibit conspicuous median fiber-attachment points. In both cases the daughter chromosomes, before separation, twist about each other.

The present study, like those of FERGUSON (6, 7) and BEAL (1), has demonstrated that the diploid number of chromosomes, each longitudinally split, is present at the metaphase of the first embryonal mitosis. The occurrence, as reported by HUTCHINSON (8), of a haploid number of paired chromosomes, each pair consisting of a single paternal and maternal member, was not confirmed. Furthermore, no evidence was seen of a transverse segmentation of chromosomes and of the passage to the poles of the segments as straight pieces. Instead, the segments separating during the anaphase, always in the form of V's or U's, are daughter chromosomes arising from a previous longitudinal splitting.

### Summary

1. In both species of pines studied, the interval between pollination and fertilization is almost exactly one year.

2. The resting nucleus of the central cell contains several nucleoli and an apparently homogeneous, coarsely granular, metaplastic substance forming an interrupted reticulum.

3. During mitosis the chromatin arises from a portion of this substance, appearing as irregular clumps that soon give rise to deeply staining, convoluted threads with a moniliform structure. These form the chromosomes, which gradually shorten and thicken. An intranuclear, multipolar, diarch spindle arises, surrounded by metaplastm not previously involved in the formation of chromosomes or the spindle itself.

4. At the equatorial-plate stage the chromosomes, 12 in number, are split longitudinally and show conspicuous median fiber-attachment points. The daughter chromosomes twist about each other, particularly when they begin to separate. They are V-shaped or narrowly U-shaped as they move toward the poles.

5. The ventral canal nucleus may or may not disorganize before reaching the resting condition. As the egg nucleus enlarges, the metaplastm reappears, soon completely obscuring the chromatin. It then exhibits nearly the same structure as the resting nucleus of the central cell. A variable number of nucleoli are present.

6. The male nucleus consists of the same kind of metaplastic substance as the egg nucleus, but contains no nucleoli. It sinks into the egg nucleus, the chromatin, differentiating in each of the conjugating nuclei after the common membrane separating them breaks down.

7. The male and female chromatin, each surrounded by its own group of spindle fibers, consists of attenuated, very slender, convoluted strands. A com-

mon multipolar, polyarch spindle is formed; this becomes diarch as the chromosomes, arising in two groups, come together.

8. The paternal and maternal chromosomes cannot be distinguished from each other when the equatorial-plate stage has been reached. As they shorten and thicken, they display prominent median fiber-attachment points and conspicuous longitudinal splitting.

9. During the metaphase of the first embryonal mitosis, 24 chromosomes are present, half derived from the male nucleus and half from the female. The longitudinal segments of each split chromosome tend to twist about each other.

10. During the anaphase, 24 V-shaped or narrowly U-shaped daughter chromosomes pass to each pole, as in any other somatic mitosis.

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# GROWTH AND DEVELOPMENT IN RANGE GRASSES. I. EARLY DEVELOPMENT OF *BOUTELOUA CURTIPENDULA* IN RELATION TO WATER SUPPLY

CHARLES E. OLMSTED

(WITH ONE FIGURE)

## Introduction

Few published data on developmental histories of range plants are available. Likewise, relatively few critical investigations of their growth and developmental responses to various ecological factors, aside from drought and grazing, have been conducted, under either natural or partially controlled environments. The accumulation and eventual systemization of such data, at least for dominant species or their ecotypes, would seem to be a necessary prelude to any further refinement in the causal interpretation of: (a) distributional data for various vegetation types; (b) the roles played by the same or different species in different parts of the range; and (c) their responses to grazing and drought.

The genus *Bouteloua*, by reason of its size, economic importance, and the differing distributional patterns and roles of its species, has been chosen for investigation. Side-oats grama, *B. curtipendula*, has recently attracted attention because of its apparent drought-resistance and increase in importance in various parts of the mixed and true prairie regions (12, 13). It has a wide range, and is thought by some "to be the most promising grama for domestication" (3). The present study traces the development of seedlings of this species in relation to water supply under a relatively controlled environment (in the greenhouse of the department of botany, University of Arizona) from November 21, 1939, to March 19, 1940. Concurrent studies on drought response and recovery by seedlings of different ages will be published in the near future.

## Methods and environmental conditions

Uniform caryopses, threshed from spikelets collected on the Santa Rita Range Reserve in 1938, were planted  $\frac{1}{4}$ - $\frac{1}{2}$  inch deep at the rate of twenty-five per pot in the air-dry soil contained in ninety-eight 2-gallon crocks. Each crock, 9 inches deep and 8 inches in diameter, and provided with a center drain, had previously been placed in position on a greenhouse bench and filled with air-dry Tumacacori coarse sandy loam soil, which had been thoroughly mixed and passed through a  $\frac{1}{2}$ -inch mesh screen. This soil, collected to a depth of 9 inches, at an altitude of 4250 feet on the Santa Rita Range Reserve, had originally supported a dense

mixed stand of perennial and annual grasses. These included *Bouteloua curtipendula*, *B. hirsuta*, *B. filiformis*, *B. aristidoides*, *Aristida divaricata*, *A. adscensionis*, and *Heteropogon contortus*. The soil is dark brown in color, has a high organic content, a pH of 6.5, a moisture equivalent of 6.42 per cent, a permanent wilting percentage of approximately 1.5, and a hygroscopic coefficient of 0.80 per cent. One surface-inch of water (824 cc.), applied to a pot of such soil, raised its average percentage moisture content to 8.25, approximately the field capacity of this soil.

Since it had been shown previously (7) that side-oats grama is sensitive to photoperiod, natural day length was supplemented by Mazda lamps to give a 16-hour light period, in an effort to simulate more closely the conditions under which seedlings of this species normally develop in southern Arizona. No accurate instruments were available for the measurement of daylight intensity. Official records<sup>1</sup> on sunshine and cloudiness show 66 per cent of the days clear and 17 per cent each of cloudy and partly cloudy days during the experiment. The cloudiness was concentrated at intervals, primarily before February 1.

A shaded hygrothermograph, placed among the pots, was operated continuously. Maximum daily air temperatures ranged from 71° to 89° F. until the middle of February, although most fell between 80° and 85° F., except on cloudy days. Maximum temperatures between 90° and 100° F. were recorded for 9 days between February 17 and March 17. Minimum daily temperatures ranged from 60° to 72°, although most were between 65° and 69° F. Relative humidity ranged 80-95 per cent through the 10 to 14-hour period during which the ventilators were closed each night, but fell to a minimum below 40 per cent for several hours in all but 17 days of the experiment.

On November 21, 1939, and again on November 24, water equivalent to 0.2 surface-inch was added to each pot with a bottle sprinkler. The pots meanwhile were covered with light paper to retard surface evaporation until the evening of November 26. Germination was excellent and uniform, and on the morning of November 27, after the application of 0.4 surface-inch of water to each, the pots were divided by randomization into series, three of twenty-six and one of twenty pots, to be watered at intervals of 3, 6, 12, and 20 days, respectively, thereafter. All pots were given 0.4 surface-inch at each watering, those continuing to the end of the experiment thus receiving a total of 15.6, 8.0, 4.4, and 2.8 surface-inches in order of decreasing watering frequency. These series will hereafter be referred to as 3's, 6's, 12's, and 20's. In table 1 is shown the minimum percentage soil moisture content at the close of certain watering intervals in each series, as determined on pots which were taken down on the various dates. The addition of 3.3 per cent to the figures will indicate the maximum average water content for

<sup>1</sup> Obtained by courtesy of Mr. H. V. SMITH.

the whole soil mass in the pots in each series during the following watering interval. Repeated observations and experiments on other pots indicated a more uniform distribution of water added to the drier soil than was expected, on the assumption that penetration downward would occur only as successive layers were wetted to field capacity. The data indicate the increase in aridity which occurred in all series in the later stages of the experiment, owing to the increasing rate of water loss by the developing plants and to the higher rate of evaporation from the soil surface due to increasing temperature and decreasing air humidity.

Because of its significance in adventitious root establishment, daily early morning observations were made on the wetness of the soil surface in each pot, as well as on the evidences of water deficit in the plants. The 3's were wet each morning until February 10, after which the surface soil was dry on the third morn-

TABLE 1

MINIMUM SOIL MOISTURE PERCENTAGE, DRY-WEIGHT BASIS, ABOVE HYGROSCOPIC COEFFICIENT, AT CLOSE OF CERTAIN WATERING INTERVALS

SERIES	DEC. 9	DEC. 17	DEC. 21	JAN. 2	JAN. 6	JAN. 14	JAN. 26
3's.....	6.51	.....	8.42	12.21	.....	7.72	5.73
6's.....	3.04	.....	3.02	4.03	.....	3.82	0.90
12's.....	2.18	.....	1.66	1.62	.....	1.67	1.12
20's.....	.....	1.51	.....	.....	1.51	.....	0.77

ing following watering until February 27, and thereafter on the second morning until the close of the experiment. Afternoon wilting appeared in this series about the middle of February on the third day of each watering interval, gradually increasing in severity and duration so that by March 2 and thereafter the plants remained wilted through the night preceding the next watering. The surface soil in the 6's was dry on the third to fifth morning of each interval until February 2, and on the second morning thereafter to the end of the experiment. Severe afternoon wilting, first recorded in this group on January 24, persisted overnight on the sixth day of the interval on February 7. Thereafter the duration of water deficit in each interval increased to a 3-day maximum of continued wilting at the close of the experiment. The 12's showed surface soil dryness by the third morning following watering until January 16, when it was dry on the second. The latter condition continued to the close of the experiment. Wilting of overnight duration gradually increased from a minimum of 2 days at the close of an interval in the first month to a maximum of 9 days out of 12 at the close. In the 20's the soil surface was dry on the second morning following watering in all intervals but the one beginning on January 6, which required 3 days for this condition to appear, thus allowing the development of the few adventitious roots which were estab-



lished in this series. Continued wilting in this series increased from 2 days at the close of the first interval to 13 days at the last. In all series the surface 1-1½ inches of soil became air-dry in 24-48 hours after the first record of morning dryness.



FIG. 1.—Upper row (left to right), 66-day old plants of *Bouteloua curtipendula* watered at intervals of 3, 6, 12, and 20 days and supplied total of 8.4, 4.4, 2.4, and 1.6 surface-inches per pot respectively; lower row, same at 118 days, and supplied total of 15.6, 8.0, 4.4, and 2.8 surface-inches.

On December 17 the plants were thinned to ten per pot, selected for uniformity. Variation obviously due to genetic differences was less than might have been expected from the mixed parent population. Variation between pots in any one series was also slight, as shown by repeated examination when pots were removed from each series for various purposes.

On December 9 and 21, January 2, 14, and 26, and March 19, plants from one

pot each of the 3's, 6's, and 12's were carefully washed out of the soil, separated from one another, and allowed to dry out in papers until they could be examined in detail. The same procedure was followed for the 20's on December 17, January 6 and 26, and March 19. Prior to further examination, the plants were soaked in water for 24 hours. Early critical examination showed that identifying characteristics of parts alive or dead when the pots were taken down were well preserved by this drying procedure, which therefore introduces no error into the data. Results presented in the following section are for the most part the averages of the measurements for the ten individuals in each pot taken down on the various dates. Figure 1 shows representative pots from each series on January 26 and March 18.

Data on adventitious root primordia include records on all which could be located by careful dissection under a binocular microscope at 14X, whether or not they had burst through the outer stem tissues or leaf bases. Only those leaves on either primary or secondary axes were counted which showed tips emergent above the surrounding leaves. Axillary buds (tillers) were located where necessary by stripping off their axillant leaves, but were counted only when they exceeded 1 mm. in length.

## Results

### PRIMARY ROOT SYSTEM

The primary root system consists only of a taproot and its branches. The primary root elongated rapidly, reaching average lengths of about 9 and 16 cm., in the 6's and 12's, 12 and 18 days after the first watering. Penetration was less in the 3's, but numerous laterals were produced in the upper 2 cm. of soil, in contrast to their absence there in the other series, where the surface soil dried rapidly. Laterals at lower depths averaged about seven per centimeter of primary root in all series, but few laterals of the second order had developed in any 18-day plants. Essentially the same stage of development was shown in the 20's when 26 days old as in the 18-day 6's and 12's, the primary root then averaging 18 cm. long. Further increases in length had occurred in the 30-day 3's, 6's, and 12's to 18 cm. in the first and to 26 cm. in the latter two series. Some 42-day plants in the 3's showed death of the entire primary root system, and it was apparently dead in all plants examined in this series at 66 and 119 days. With adequate establishment of adventitious roots, it probably becomes nonfunctional when 6-10 weeks old, although the actual cause of death in the 3's may have been lack of aeration during the period of excessive soil moisture centering around January 2 (table 1). Unexpected death of adventitious roots also occurred in the 3's at this time. In the 6's, all primary roots examined were alive at 42 days and dead at 66. At 119 days, however, they were still alive on two plants with one or no

established adventitious roots, but were dead on all those with an adequate secondary root system. In most of the 12's and 20's, the primary roots apparently remained alive until the close of the experiment, and in length and degree of branching in individual plants showed a strong inverse correlation with their numbers of functional adventitious roots.

### PRIMARY STEM AND LEAVES

Seventy-two hours after the initial watering, tips of the coleoptiles were emergent 5-10 mm. above the soil surface. Twenty-four hours later, elongation of the first internode (mesocotyl) had ceased, bringing the coleoptilar node to the ground line, or in a few cases as much as 8 mm. above it. These latter plants, if among

TABLE 2  
AVERAGE GROWTH DATA ON THINNED PLANTS, 26 DAYS OLD  
DECEMBER 17

	SERIES			
	3's	6's	12's	20's
Total number of plants.....	152	188	152	98
Surface-inches water supplied.....	3.2	2.0	1.2	0.8
Number, primary foliage leaves.....	3.6	3.5	3.2	2.9
Length to tip, cm.....	12.0	11.1	9.1	6.0
Dry weight of top, mg.....	10.1	7.9	5.5	3.3
Visible adventitious roots.....	1.3	1.0	0.8	0.4

the 6's, 12's, or 20's, rarely succeeded in establishing adventitious roots. On the fourth or fifth day the first foliage leaf burst through the coleoptile, by the end of the sixth had attained a length of 25-50 mm., and on December 3, 12 days after first watering and 3 days after the beginning of divergence in treatment, was 60-90 mm. long in all series. The second leaf tip also emerged in most plants on December 3.

The effect of variation in water supply on growth and development of tops, beginning to be noticeable on December 9, as shown by data for one pot in each series in table 3, was marked by December 17, the seventeenth day following divergence in treatment, as seen in table 2, where the data are based on all plants removed in thinning. Growth rates in this species in this soil are obviously affected by decreasing soil moisture before the latter has fallen to the level of the permanent wilting percentage, which was not reached in any of the pots in the early stages of the experiment.

The rate of increase in foliage leaf number with age, and of plant height, as measured by distance from the ground to tip of the leaf uppermost when all were

extended, is shown in table 3. Variation in water supply, within the range of this experiment, apparently had less effect on the number of leaves produced by the primary axis than upon plant height. The latter was affected by differences in culm elongation, as well as by differences in leaf size, produced through the dwarfing effect of deficient water supply in the drier series. The number of basal internodes which matured without elongating, and the age at which elongation began, as well as the number and length of expanded segments, were all affected.

TABLE 3  
PLANT HEIGHT,\* TOTAL NUMBER OF PRIMARY LEAVES, AND OF DEAD  
OR DYING PRIMARY LEAVES

SERIES	DATE AND AGE IN DAYS							
	DEC. 9 (18)	DEC. 17 (26)	DEC. 21 (30)	JAN. 2 (42)	JAN. 6 (46)	JAN. 14 (54)	JAN. 26 (66)	MAR. 19 (119)
3's								
Height, cm.....	5.9	.....	14.0	27.9	.....	34.0	53.8	64.6
No. of leaves.....	3.1	.....	4.1	5.9	.....	7.4	8.6	11.8
Dead or dying leaves..	0.0	.....	1.2	4.2	.....	5.6	6.4	10.4
6's								
Height, cm.....	7.0	.....	12.4	23.6	.....	35.6	44.4	43.0
No. of leaves.....	3.0	.....	4.4	5.7	.....	6.7	7.9	10.0
Dead or dying leaves..	0.0	.....	0.9	3.3	.....	4.7	6.6	9.5
12's								
Height, cm.....	5.4	.....	8.7	23.3	.....	27.5	30.7	34.9
No. of leaves.....	2.2	.....	4.5	5.7	.....	6.5	7.1	9.1
Dead or dying leaves..	0.0	.....	0.3	3.1	.....	4.2	5.4	9.0
20's								
Height, cm.....	.....	6.1	.....	.....	16.2	.....	21.4	28.7
No. of leaves.....	.....	3.0	.....	.....	5.3	.....	6.9	8.7
Dead or dying leaves..	.....	1.1	.....	.....	2.6	.....	6.2	8.5

\* Measured from ground line to tip of leaf uppermost when all were extended.

At 66 days the average numbers of elongated or elongating internodes per plant with decreasing watering frequency were 1.4, 1.3, 0.2, and 0, the completely mature units averaging 58, 45, and 43 mm. in length, respectively. Comparable data at 119 days are 5.1, 3.0, 1.25, and 0.5, with lengths of 72, 46, and 42 mm. No elongated internodes were completely mature in the 20's. The usual lowest internode to elongate (considering the mesocotyl as the first internode) was the seventh in the 3's, seventh or eighth in the 6's, and eighth or ninth in the 12's and 20's. Most 20's, so far as could be determined, thus had one or two more unelongated internodes per plant than had the 3's. This change in position for internodal elongation of the culm, apparently due (at least indirectly) to deficient water supply, is probably the most fundamental morphogenetic effect induced by the treat-

ment, for it seems improbable that the observed differences are the result of inadequate sampling. It should be noted that maturation of the leaf base and basal internode regions was complete in nearly all plants examined only two or three nodes below the apex of the axis in all series and ages.

The successive dying of the four or five lowest foliage leaves, which occurred in all series in 4-8 weeks, was apparently a normal process, probably caused by the rupture of the vascular strands in their bases through the development of adventitious roots and axillary tillers, and did not seem due directly, except possibly in the 20's, to any deficit in external water supply. The death of leaves above this level (table 3), and of those of the secondary axes (table 5), was hastened in all series in the last 6 weeks by the increasing aridity of aerial environment and by the increasing difficulty for the larger plants to maintain a favorable water balance with the amount supplied. In the 3's and 6's especially, the developing tillers undoubtedly diverted water necessary to maintain the upper leaves on the elongating primary axis. This increasing deficit in upper portions of the plants in these series may likewise have been responsible for their failure to initiate spikes at an age and apparent stage of development when such initiation might have been expected. It is known (2, 11) that individuals of this perennial will flower within the first four months of growth; but the complex interactions of age of plant, photoperiod, and temperature known to affect reproductive activity in certain grasses (10) are almost completely unknown for this species. The terminal growing point was still alive in all plants at the close of the experiment.

#### TILLER DEVELOPMENT

Axillary bud primordia successively appeared in the axils of all primary leaves except the scutellum, coleoptile, and the upper two or three foliage leaves in the 119-day 3's and 6's. Tables 4 and 5 show the total number of buds, the size attained, the number of visible leaves, and the death of successive leaves in the various tillers formed from these buds, in relation to age and watering frequency. The data emphasize the relatively greater influence of water supply upon size than upon the numbers of organs differentiated, also noted for the primary axis. In all series the ratio of leaves per tiller to its length shows a tendency to decrease with increase in nodal position. This is most obvious on the older tillers where a larger proportion of both leaves and internodes had ceased elongating. Occasional plants appeared in all series in which the basal axillary bud did not elongate. These account in part for the lesser average height of the first tiller, as compared with the second or third, in the older plants. These basal buds often assumed a horizontal position, in contrast to the apogeotropic tendencies of all others; but at the close of the experiment it was impossible to determine whether they would remain dormant or become rhizomatous. Relatively short rhizomes

are characteristic of this perennial species. It was likewise undetermined whether or not this failure of basal buds to enlarge was related to depth of planting, as it seems to be in wheat (4).

The largest tillers in each series at 119 days occurred most commonly in the axil of the second, occasionally of the third, foliage leaf (fourth and fifth nodes). They averaged 46.3, 29.9, 20.3, and 12.1 cm. in length, with leaf numbers of 9, 8.5, 7.9, and 7.7 in order of decreasing water supply. At the same age, and in the

TABLE 4  
TOTAL NUMBER OF PRIMARY, SECONDARY, AND TERTIARY BUDS

SERIES	DATE AND AGE IN DAYS						
	DEC. 9 (18)	DEC. 17 (26)	DEC. 21 (30)	JAN. 2 (42)	JAN. 6 (46)	JAN. 26 (66)	MAR. 19 (119)
3's							
Primary.....	0	.....	0.7	3.3	.....	6.0	7.4
Secondary.....	0	.....	0	0.6	.....	15.2	25.0
Tertiary.....	0	.....	0	0	.....	2.2	24.8
6's							
Primary.....	0	.....	0.4	2.8	.....	5.4	7.4
Secondary.....	0	.....	0	0.2	.....	9.6	21.8
Tertiary.....	0	.....	0	0	.....	0.9	13.7
12's							
Primary.....	0	.....	0.1	2.9	.....	5.0	7.1
Secondary.....	0	.....	0	0	.....	4.5	17.6
Tertiary.....	0	.....	0	0	.....	0	9.0
20's							
Primary.....	.....	0	.....	.....	2.4	4.1	6.6
Secondary.....	.....	0	.....	.....	0	2.2	12.8
Tertiary.....	.....	0	.....	.....	0	0	0

same order, the average numbers of primary tillers over 15 mm. in length were 5.4, 4.7, 4.2, and 3.0. Thus in the 3's and 6's practically all buds borne on the basal portion of the axis had expanded, in contrast to less actual and much lower percentage development in the 12's and 20's with their greater number of basal nodes. In the layman's language the latter had failed to "stool out well," due primarily to a retardation in growth. Internodal elongation in primary tillers had occurred after 119 days in 3's, 6's, and 12's, an average of 3.0, 1.4, and 0.7 tillers per plant showing elongation in the respective series. Position of the first elongating internode in the secondary axis decreased from 6-9 to 4-6 in passing from the first to fourth primary tiller. Positive correlation of its position with water supply is not possible with the data now available.

The retardation in differentiation due to water deficit shows most clearly in the data on secondary and tertiary buds (table 4). Secondary buds were regularly

TABLE 5  
 LENGTH OF TILLERS, AND TOTAL NUMBERS OF LEAVES AND OF DEAD AND  
 DYING LEAVES, ON SUCCESSIVE PRIMARY TILLERS

SERIES	DEC. 21	JAN. 2	JAN. 6	JAN. 26	MAR. 19
	1ST TILLER				
3's					
Length, mm.....	5*	43	.....	282	337
No. of leaves.....	1.4*	4.9	.....	8.8	10.2
Dead or dying leaves.....	0	0	.....	6.0	9.2
6's					
Length, mm.....	3	27	.....	116	134
No. of leaves.....	1.0	3.6	.....	7.1	8.8
Dead or dying leaves.....	0	0	.....	3.8	8.3
12's					
Length, mm.....	3	14	.....	44	86
No. of leaves.....	1.0	3.5	.....	6.1	6.9
Dead or dying leaves.....	0	0	.....	2.1	5.8
20's					
Length, mm.....	.....	.....	7.7	50	60
No. of leaves.....	.....	.....	3.6	6.0	7.3
Dead or dying leaves.....	.....	.....	0	1.1	6.9
	2ND TILLER				
3's					
Length, mm.....	5	31	.....	311	446
No. of leaves.....	1.5	3.9	.....	7.4	9.6
Dead or dying leaves.....	0	0	.....	5.0	8.6
6's					
Length, mm.....	0	29	.....	250	283
No. of leaves.....	0	3.0	.....	6.3	8.6
Dead or dying leaves.....	0	0	.....	4.2	8.3
12's					
Length, mm.....	0	18	.....	103	175
No. of leaves.....	0	2.8	.....	5.9	8.4
Dead or dying leaves.....	0	0	.....	2.0	8.0
20's					
Length, mm.....	.....	.....	16	68	111
No. of leaves.....	.....	.....	3.0	5.2	7.9
Dead or dying leaves.....	.....	.....	0	1.1	7.1

\* Based on average total tillers present in ten plants.

TABLE 5—Continued

SERIES	DEC. 21	JAN. 2	JAN. 6	JAN. 26	MAR. 19
	3RD TILLER				
3's					
Length, mm.....		7		251	408
No. of leaves.....		1.8		6.4	8.0
Dead or dying.....		0		4.4	7.2
6's					
Length, mm.....		8		211	236
No. of leaves.....		1.4		5.3	8.0
Dead or dying leaves.....		0		2.7	7.1
12's					
Length, mm.....		5		68	194
No. of leaves.....		1.3		4.6	7.6
Dead or dying leaves.....		0		0.8	7.0
20's					
Length, mm.....			4	32	101
No. of leaves.....			1.1	3.9	6.9
Dead or dying leaves.....			0	0.1	6.4
	4TH TILLER				
3's					
Length, mm.....		3		191	178
No. of leaves.....		1.0		5.0	7.0
Dead or dying leaves.....		0		1.8	6.8
6's					
Length, mm.....		3		115	184
No. of leaves.....		1.0		4.4	6.2
Dead or dying leaves.....		0		0.5	5.6
12's					
Length, mm.....				22	140
No. of leaves.....				3.3	6.2
Dead or dying leaves.....				0.1	5.6
20's					
Length, mm.....				5	25
No. of leaves.....				1.3	3.8
Dead or dying leaves.....				0	2.7



TABLE 6

AVERAGE NUMBER PER PLANT, BY SIZE CLASSES AND CONDITION, OF ADVENTITIOUS  
ROOTS AND PRIMORDIA IN RELATION TO AGE AND WATER SUPPLY

SERIES	LENGTH (MM.)	DATE AND AGE IN DAYS						
		DEC. 9 (18)	DEC. 17 (26)	DEC. 21 (30)	JAN. 2 (42)	JAN. 6 (46)	JAN. 26 (66)	MAR. 19 (110)
3's								
Living.....	50+	0.2	.....	0.8	2.2	.....	8.8	10.8
Living.....	20-50	0.4	.....	0.5	0.8	.....	4.4	0
Living.....	5-20	0.5	.....	0.6	1.6	.....	5.8	0
Living.....	5-	0.3	.....	0.7	1.6	.....	4.2	15.6
3's								
Dead.....	50+	0	.....	0	0.1	.....	0	0.8
Dead.....	20-50	0	.....	0.1	0.3	.....	0.6	2.4
Dead.....	5-20	0	.....	0.1	0.4	.....	0.4	6.0
Dead.....	5-	0	.....	0.2	1.4	.....	0.8	7.8
Total 3's living and dead.....		1.4	.....	3.0	8.4	.....	25.0	43.4
6's								
Living.....	50+	0	.....	0.5	2.2	.....	6.0	5.9
Living.....	20-50	0	.....	0.1	0.2	.....	0.1	0
Living.....	5-20	0	.....	0.4	0.3	.....	0.6	0.3
Living.....	5-	0.3	.....	1.0	0.4	.....	0	11.7
6's								
Dead.....	50+	0	.....	0	0	.....	0.4	0.3
Dead.....	20-50	0	.....	0.2	0.1	.....	0.6	1.1
Dead.....	5-20	0	.....	0.1	0.4	.....	1.0	7.6
Dead.....	5-	0	.....	0.1	1.3	.....	4.6	12.9
Total 6's living and dead.....		0.3	.....	2.4	4.9	.....	13.3	39.8
12's								
Living.....	50+	0	.....	0	2.1	.....	2.6	3.9
Living.....	20-50	0	.....	0	0.4	.....	0.4	0
Living.....	5-20	0	.....	0.5	0.2	.....	0.2	0
Living.....	5-	0	.....	0.5	0.9	.....	0.1	14.2
12's								
Dead.....	50+	0	.....	0	0	.....	0	0
Dead.....	20-50	0	.....	0	0	.....	0.3	0.4
Dead.....	5-20	0	.....	0	0.2	.....	1.5	3.1
Dead.....	5-	0	.....	0.4	0.1	.....	7.1	5.3
Total 12's living and dead.....		0	.....	1.4	3.9	.....	12.2	26.9
20's								
Living.....	50+	.....	0	.....	.....	0.1	1.5	1.7
Living.....	20-50	.....	0	.....	.....	0	0	0
Living.....	5-20	.....	0	.....	.....	0	0	0
Living.....	5-	.....	0.5	.....	.....	0	1.3	9.5
20's								
Dead.....	50+	.....	0	.....	.....	0	0	0
Dead.....	20-50	.....	0	.....	.....	0	0.4	0
Dead.....	5-20	.....	0	.....	.....	0.7	1.0	1.6
Dead.....	5-	.....	0	.....	.....	2.9	5.5	7.3
Total 20's living and dead.....		.....	0.5	.....	.....	3.7	9.7	20.1

differentiated in the axils of all secondary leaves except the prophylls, the lowest three or four (occasionally all) leaves of the lowest tillers which failed to elongate, and some of the uppermost leaves on elongated secondary axes. Buds were sometimes found in the axils of prophylls, especially those on the higher secondary axes. No positive correlation of this occurrence with water supply was evident. At the end of 4 months the number of secondary tillers over 15 mm. in length was low, averaging only 5, 2.6, and 2.9 in the 3's, 6's, and 12's, respectively. Tertiary buds grew only slightly in any of the plants possessing them, and buds of the fourth order were found rarely in the 3's and 6's at 119 days. The greatest number of tertiary buds occurred on the second primary tiller, in accord with its usual greater size and with the usual location of the largest secondary tillers on each plant.

#### SECONDARY ROOT SYSTEM

The apparent normal plan of adventitious root initiation in this species involves the differentiation of one primordium at the coleoptilar or second node, at the level of insertion of the coleoptile, and on the same side of the axis and in the same plane as the line along which the coleoptile splits. Successive nodes, up to the first or second below the first elongating internode, normally produce a pair of primordia on the side of the axis opposite the bud insertion at the same node. These usually emerge just at or slightly below the level of leaf insertion, and above and slightly to either side of the next lower bud, so that in development they grow down on opposite sides of, and sometimes almost surround, this secondary axis. Roots from the third node usually surround the split coleoptile in similar fashion. An additional pair of primordia are normally initiated slightly above the base of the first elongating internode or on the internode just below it when none are produced on the last node of the abbreviated stem. These usually emerge on the same side of the axis as the axillary bud, but slightly above and to either side of the latter. A third primordium is sometimes found at the same level but directly opposite the point of insertion of the bud. To simplify tabulation, these last two or three roots are listed in table 7 under the number of the node next above them.

The actual number of root primordia produced by the primary axis rarely agrees with this normal plan. Two tendencies, sometimes working together and sometimes in opposite directions, seem to be superimposed upon this normal development. With greater or less supplies of water, a greater or less number of primordia tend to be associated with any node, as may be seen by comparing the totals in the different series for the third, fourth, and fifth nodes of the primary axis (table 7). Numbers initiated on the higher nodes, however, and on secondary axes as well, in individual plants vary inversely with the number of primordia which have already matured into functional roots. The effect of this latter tend-

TABLE 7

ADVENTITIOUS ROOT AND PRIMORDIA NUMBERS, CONDITION, AND DISTRIBUTION ON AXIS, BY SIZE CLASSES, ON 110-DAY PLANTS

SERIES	LENGTH (mm.)	PRIMARY STEM AXIS: ROOTS PER NODE									SECONDARY STEM AXES: ROOTS PER AXIS					TER- TIARY AXES: TOTAL ROOTS	GRAND TOTAL	
		Node 2*	3	4	5	6	7	8	9	Total	Axis 1	2	3	4	5			Total
3's Living..... Living..... Living..... Living.....	50+	0.8	1.4	0.8	1.4	1.2	0.2	0	0	5.8	1.8	2.0	0.8	0	0	4.6	0.4	10.8
	20-50	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	5-20	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	5-	0	0	0	0	0	0.2	0.6	0	0.8	3.2	2.2	2.6	2.2	0.8	11.0	3.8	15.6
3's Dead..... Dead..... Dead..... Dead.....	50+	0	0	0	0.2	0.2	0	0	0	0.4	0.2	0.2	0	0	0	0.4	0	0.8
	20-50	0	0.2	0.8	0.6	0	0.2	0	0	1.8	0	0.4	0	0	0	0.4	0.2	2.4
	5-20	0	0.2	0.6	0.6	0	0.4	0	0	1.8	1.8	0.8	0.8	0.4	0	3.8	0.4	6.0
	5-	0.2	0.8	0.4	0	0.4	0.8	0.6	0	3.2	1.6	1.2	0.6	0.6	0.2	4.2	0.4	7.8
Total living and dead.....		1.0	2.6	2.6	2.8	1.8	1.8	1.2	0	13.8	8.6	6.8	4.8	3.2	1.0	24.4	5.2	43.4
6's Living..... Living..... Living..... Living.....	50+	0.3	0.5	0.9	0.8	0.5	0.3	0	0	3.3	0.8	1.1	0.6	0	0	2.5	0.1	5.9
	20-50	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	5-20	0	0	0	0	0	0	0	0	0	0	0.1	0.1	0.1	0	0.3	0	0.3
	5-	0	0	0	0	0	0.4	0.8	0.7	1.9	1.1	2.0	2.3	1.5	0.3	7.2	2.6	11.7
6's Dead..... Dead..... Dead..... Dead.....	50+	0	0	0.1	0.2	0	0	0	0	0.3	0	0	0	0	0	0	0	0.3
	20-50	0	0.1	0.3	0.3	0	0.2	0	0	0.9	0	0.2	0	0	0	0.2	0	1.1
	5-20	0.4	0.5	0.7	0.7	1.6	0.3	0	0	4.2	1.0	1.6	0.4	0.2	0	3.2	0.2	7.6
	5-	0.3	0.6	0.1	0.4	1.0	1.8	0.3	0.4	4.9	2.6	2.0	2.3	0.6	0	7.5	0.5	12.9
Total living and dead.....		1.0	1.7	2.1	2.4	3.1	3.0	1.1	1.1	15.5	5.5	7.0	5.7	2.4	0.3	20.9	3.4	39.8

\* Coleoptilar node.

TABLE 7—Continued

SERIES	LENGTH (MM.)	PRIMARY STEM AXIS: ROOTS PER NODE										SECONDARY STEM AXES: ROOTS PER AXIS					TER- TIARY AXES: TOTAL ROOTS	GRAND TOTAL
		Node 2*	3	4	5	6	7	8	9	Total	Axis 1	2	3	4	5	Total		
12's†																		
Living.....	50+	0	0.5	1.25	0.875	0.75	0	0	0	3.37	0.25	0.25	0	0	0	0.5	0	3.9
Living.....	20-50	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Living.....	5-20	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Living.....	5-	0	0	0	0	0	0.63	1.75	0.125	2.5	2.13	3.13	3.0	1.88	0.125	10.25	1.5	14.2
12's†																		
Dead.....	50+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Dead.....	20-50	0.125	0	0	0	0.25	0	0	0	0.38	0	0	0	0	0	0.875	0	0.4
Dead.....	5-20	0.375	0.25	0.25	0.625	0.5	0.25	0	0	2.25	0.25	0.5	0.125	0	0	0.875	0	3.1
Dead.....	5-	0.5	0.5	0.25	0.5	0.5	1.13	0.375	0	3.75	0.5	0.5	0.375	0	0	1.375	0.125	5.3
Total living and dead.....		1.0	1.25	1.75	2.0	2.0	2.0	2.13	0.12	12.25	3.125	4.375	3.5	1.875	0.125	13.0	1.625	26.9
20's																		
Living.....	50+	0	0.2	1.0	0.5	0	0	0	0	1.7	0	0	0	0	0	0	0	1.7
Living.....	20-50	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Living.....	5-20	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Living.....	5-	0	0	0	0	0.4	1.8	1.5	0.3	4.0	1.6	2.1	1.8	0	0	5.5	0	9.5
20's																		
Dead.....	50+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Dead.....	20-50	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Dead.....	5-20	0.3	0.2	0.1	0.6	0.1	0.1	0	0	1.4	0.2	0	0	0	0	0.2	0	1.6
Dead.....	5-	0.7	1.3	0.7	1.0	1.5	0.5	0.2	0	5.9	0.6	0.7	0.1	0	0	1.4	0	7.3
Total living and dead.....		1.0	1.7	1.8	2.1	2.0	2.4	1.7	0.3	13.0	2.4	2.8	1.9	0	0	7.1	0	20.1

† Averaged from eight plants.

ency can be seen most strikingly in the totals for the sixth and seventh nodes in the 6's, and for the sixth node in the 20's (table 7). The higher average values for these nodes in these series as compared with the 3's and 12's were due almost entirely to the numbers of roots initiated at these levels in the first pair of series by one or two plants per pot which had thus far failed to establish any adventitious roots. The tendency was suppressed in the 20's to a greater extent than in the 6's, probably by the greater inadequacy of the water supply.

Because of the abbreviated character of the basal internodes of the primary stem, it was impossible, by dissection alone, to determine the nodal or internodal character of all the roots produced in excess of the normal. Those which seemed definitely to be internodal<sup>2</sup> were included with those emerging from the next higher node, in tabulation. No attempt was made to work out the plan, if any, in the position of these roots, nor in that of those produced on secondary and tertiary axes. The curved character and the exceedingly abbreviated basal internodes of the branch axes make such analysis extremely difficult. The first root to appear, and the one which most commonly became established, on the secondary shoots, emerged on the lower side of the stem, and thus at right angles to the plane of phyllotaxy, at the third or fourth node.

The rate of production of primordia and the total number produced naturally varied with the rate and degree of development of the shoots, as related to water supply, as may be seen from the totals in table 6, and in detail in table 7. Scatter charts, based on the analysis of individual plants, showed a strong correlation between totals of root primordia produced and development of tops, as expressed in total numbers of axillary buds. The various treatments had little apparent effect on this relationship. Correlation was less apparent between total bud numbers and total live roots over 50 mm. long, but was more evident, although still weak, when tillers over 15 mm. long were correlated with the same root condition. This possibly indicates again that water supply for the entire plant, if it is related to the number of functional roots, has less effect upon numbers of parts differentiated in the top than upon their size. In this connection, no apparent correlation was found in any series between the size and development of tillers at any specific node and the number of functional roots established at the same or adjacent nodes. This might have been expected in view of the known irregularity (1) in the connection of the vascular elements of roots and buds in certain grasses with those of their parent axis at the same nodal level.

The ultimate development of any primordium seems to be intimately connected with the water supply, both in the plant and in the environment, during a varying length of time following its initiation, and with its position above the soil surface. The data in table 7 show the condition, when the plants were 4

<sup>2</sup> ARBER (1) notes rare exceptions to the common interpretation that all adventitious grass roots are nodal in origin.

months old, of the roots which had been initiated successively at the different nodes of the primary axis. As an example, initiation of the root at the coleoptilar node probably took place in all series when the plants were about 15 days old. In the 3's a continuously moist soil surface and an abundance of water in the plant allowed growth to proceed promptly in most cases, and new primordia began to form at the third node, resulting at 18 days (on December 9) in the live root condition shown in table 6. Table 7 shows that eight of the ten plants examined at 119 days in this series had functional roots on the coleoptilar node. In the 6's and 12's at 18 days, with no water added for 6 and 12 days, respectively, the primordia were so small as not to be counted in most cases, as shown by the data in table 6 for December 9. These primordia responded immediately to the addition of water on this date, some of them reaching lengths of 10 mm. over night. But the rate of soil drying, downward, exceeded their rates of growth, and only three of the 6's and none of the 12's show roots established from this node (table 7). In the 20's the equivalent primordia were delayed in their growth even longer following initiation, although some had protruded through the stem tissue about 1 mm. at 26 days. They all grew immediately upon watering at this time, some reaching lengths of 5 mm. within 6 hours; but in spite of this rapid growth, none succeeded in becoming established before the surface soil again became dry.

In 6's, 12's, and 20's, each application of water invariably resulted in immediate elongation of the small primordia already initiated, until the plants were about 8-10 weeks old. After this, and earlier in the concurrent studies on drought effect and recovery, a decided lag (often 2 or 3 days) occurred after watering before there was much evidence of growth in primordia already initiated. The latter condition was also found in older plants of all series where root tips, deep in the soil, had ceased to grow in the later stages of a watering interval and had become differentiated almost to the tip. This lag in resumption of growth by the primordia of older plants, which likewise seem to differentiate to the tip if growth is delayed, increases their difficulty in establishment in a soil which rapidly dries after water is added, and accounts in part for the large number of dead roots under 20 mm. long in all series on March 19.

The 3's show a lower number of established roots than might be expected. Death losses to account for this were due probably to the possible lack of aeration during late December and January, as mentioned previously, possibly accompanied by fungus or bacterial invasion. The death of roots initiated at a later period in the 3's, and that of all roots in the other series, seemed to be due to desiccation. The somewhat higher percentage of living long roots in the 20's than was anticipated was due entirely to the coincidence of a 4-day period of cloudiness following the watering at 46 days, allowing some of the primordia from the third, fourth, and fifth nodes to become established before the soil surface became dry (table 7).

### Discussion

The more obvious effects of varying water supply upon the size and development of *Bouteloua curtipendula* need little interpretation. The primary root showed, under the relatively mesic conditions of the early part of this experiment, the rapid and deep penetration characteristic of many species adjusted to semi-arid or arid environments (2), so necessary for their successful establishment. Its future development and persistence seem closely related to the establishment of adventitious roots. It apparently becomes nonfunctional in plants where abundant and frequent renewal of water supply allows the latter to develop, but persists for at least 4 months as the sole or most important part of the absorbing system in the drier series where few or no nodal roots become functional. No assumptions are made as to the causes of death or persistence, except in the 3's, where death may have been due to inadequate aeration. It has been reported that winter wheat (8) may complete its life cycle with only the seminal roots established; and McCALL (9) states that, unless destroyed in some way, the primary root system remains functional throughout the life of the wheat plant. The latter would not be true in perennial side-oats grama, and it is doubtful whether, under natural conditions, its mesocotyl is strong enough to withstand, alone, the mechanical stresses imposed upon plants of the size attained by the 20's at the end of 4 months (fig. 1), even though sufficient water and nutrients might be absorbed and transmitted by the primary root system to maintain life.

The position of the coleoptilar, or first crown, node at the ground surface (probably a usual condition in nature with shallow planting) would seem to be disadvantageous for a species growing in arid environments, for each successively formed nodal root primordium to become functional must surmount the difficulties encountered in growing through an inch or two of surface soil which is dry much of the time. Notwithstanding the relatively rapid growth rates of such primordia when water became available, it apparently required about 3 consecutive days of soil surface wetness for successful establishment of such roots. Such conditions are occasionally part of the natural environment of this species during the critical periods following its germination, but a very high percentage of seedlings in nature probably do not survive because of failure in nodal root establishment, especially in widely spaced bunchgrass types. In older plants the increasingly mesic conditions provided by the plant's own shade should aid establishment of roots from tillers, while in propagation from rhizomes this situation probably is met easily. Development has not been traced this far, and no references have been found to the relative position of root origin on plants so produced.

The relative importance of primary and secondary root systems in the early life of the whole plant is obvious where one dies or the other fails in establishment.

No effort was made, however, to isolate the activities of each system, aside from death or persistence, under different conditions of water supply. Plants with no nodal roots were retarded in growth as compared with plants bearing functional adventitious roots in the same pots, but no obvious effect upon the relative size or differentiation of the primary axis as compared with the tillers was noticeable in relation to the presence or absence of one or the other systems. Such effects have been recorded by KRASSOVSKY (5) in spring wheat, barley, and rye. She concluded that the primary stem is supplied mainly by the seminal roots; the tillers, by the nodal roots. Since retardation in floral initiation was found in both primary stems and tillers in the present experiment, as compared with a 124-day plant illustrated by WEAVER (11), the failure of the main axis to flower at this age probably is not related causally, or only indirectly, to the death of the primary roots in the wetter series.

No explanation is advanced for the greater number of unelongated basal internodes in the primary stems of the dry series as compared with the wet, nor for the tendency of root primordia to develop in greater numbers on the lower nodes of the axis in the wet series than in the dry. The tendency toward increased root primordia numbers on the higher nodes in any series where roots from the lower nodes have failed in establishment possibly is related to the mechanism responsible for the rapid development of adventitious roots by the shoots of certain germinating grass embryos deprived of their seminal roots (6). LA RUE did not state specifically, however, whether or not the number of roots produced by any specific portion of the axis in his experiments was greater than it would have been under normal development at a later date. Regardless of the causal mechanism, the increased production of primordia noted in the present experiment, if followed by successful establishment at a favorable time, should be of adaptive significance in compensating for the earlier failure.

Detailed discussion of the implications of the data from this experiment in relation to the topics suggested in the introduction will be deferred until experiments with this species in other partially controlled environments are completed.

### Summary

1. The growth of seedlings of *Bouteloua curtipendula* in a coarse sandy loam soil, in 2-gallon crocks 9 inches deep, watered at intervals of 3, 6, 12, and 20 days, was followed for 4 months. All plants received supplementary illumination to provide a 16-hour photoperiod.

2. At the end of the experiment, plant height, and total numbers of: leaves and nodes, and elongated internodes on the primary axis; primary, secondary, and tertiary tillers; adventitious root primordia differentiated; primordia differentiated on lower nodes of primary axis; and living functional adventitious roots



all were correlated directly with the amount of water supplied, although to different degrees. Numbers of: mature basal unelongated internodes; basal nodes bearing roots; and root primordia on upper crown nodes were correlated inversely with water supply, to a limited degree.

3. Height or length, as measured to tips of leaves, of both primary and secondary axes, was much more affected by water supply than was the number of leaves, buds, or roots differentiated on corresponding axes.

4. No plants flowered, although primary culm elongation began in the 3's and 6's within 60 days and had begun in the 20's at 4 months.

5. Both size and numbers of axes of the third and fourth order were much lower in the drier series than in the wetter ones.

6. The primary root system developed rapidly, but consisted only of a single taproot and its branches. It became nonfunctional in most 3's and 6's within 2 months, but remained alive in 12's and 20's until the close of the experiment, and in some cases persisted as the only functional part of the root system.

7. Adventitious roots require approximately 3 consecutive days of surface soil wetness to penetrate sufficiently to become established. A tendency was noted for a relative increase in the numbers of primordia differentiated, at the upper crown nodes and on tillers, by plants on which no nodal roots had become established.

The writer wishes to express his appreciation for the facilities afforded this investigation in the department of botany, University of Arizona, and for aid extended in various ways by Dr. D. M. CROOKS and Dr. R. A. DARROW. Assistance was also provided at various times by Drs. W. G. MCGINNIES, E. L. LITTLE, JR., and Mr. K. W. PARKER, all of the Southwestern Forest and Range Experiment Station.

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# FURTHER OBSERVATIONS ON FACTOR Z<sup>1</sup>

WILLIAM J. ROBBINS

(WITH FOUR FIGURES)

## Introduction

In earlier publications (2, 3, 4, 5) it was proposed that an unidentified factor, called for convenience factor Z, favorably influences the spore germination, early mycelial growth, and gametic reproduction of *Phycomyces blakesleeana*s. Factor Z appeared to be present in extracts of many natural products, including those of potato tubers. ROBBINS and HAMNER (5) separated an extract of potato tubers into two fractions by treatment with charcoal. One fraction, Ca, was adsorbed on charcoal and eluted with ammoniacal acetone; the other, Dr, was the charcoal filtrate. Each fraction acted favorably upon the development of *Phycomyces* in a medium containing thiamin, but the effect of the two fractions when combined in suitable proportions was greater than would be anticipated from their effects when used singly. ROBBINS and HAMNER suggested that the potato extract contained at least two unidentified factors: Z<sub>1</sub>, adsorbed by charcoal and eluted by ammoniacal acetone and Z<sub>2</sub>, present in the filtrate of potato extract which had been treated with charcoal. In the present paper further observations on these factors are reported.

## Material and methods

Two basic media were used. Solution 1 contained per liter: 50 gm. dextrose, 1.5 gm. KH<sub>2</sub>PO<sub>4</sub>, 0.5 gm. MgSO<sub>4</sub> · 7 H<sub>2</sub>O, 0.5 mg. thiamin, and asparagine as indicated. Solution 2 contained per liter: 100 gm. dextrose, 15 gm. KH<sub>2</sub>PO<sub>4</sub>, 5 gm. MgSO<sub>4</sub> · 7 H<sub>2</sub>O, 0.5 mg. thiamin, and asparagine as indicated. To each of these solutions the following trace elements were added: 0.005 p.p.m. B, 0.02 p.p.m. Cu, 0.1 p.p.m. Fe, 0.01 p.p.m. Ga, 0.01 p.p.m. Mn, 0.01 p.p.m. Mo, and 0.09 p.p.m. Zn. All inorganic chemicals were of chemically pure grade: the dextrose was Corn Products Company chemically pure; the thiamin was Merck's synthetic; the asparagine was purified by precipitation from alcohol. For liquid cultures, 25 ml. of solution were used in 125-ml. Erlenmeyer flasks; for agar cultures, 25 ml. of media in petri dishes 90 mm. in diameter and 10 mm. deep were used. All solutions were sterilized at 110° C.; the temperature of incubation was 25° ± 1° C.; dry weights were determined by drying at 100° C.

<sup>1</sup> Assistance in this work was furnished by the personnel of Works Projects Administration, Official Project 65-1-97-23, W.P. 5.

EFFECT OF CA AND DR FRACTIONS ON PHYCOMYCES AND A METHOD FOR  
APPROXIMATING FACTORS  $Z_1$  AND  $Z_2$  IN SOLUTIONS  
OF UNKNOWN COMPOSITION

Drops of a spore suspension of *Phycomyces* were placed on 25 ml. of solution 1 solidified with 1 per cent agar. A similar procedure was followed with the same medium to which the Dr fraction, the Ca fraction, or combinations of the two were added (table 1). After 12 hours of incubation at 25° C. the percentage of germinated spores was determined. The Ca fraction contained 5.08 mg. solids per ml., and 1 ml. represented the extract from 50 gm. of potato. The Dr fraction contained 241.6 mg. per ml., and 1 ml. represented the extract from 50 gm. of potato. The preparation of these two fractions has been described earlier (5).

TABLE 1  
EFFECT OF CA AND DR FRACTIONS ON PERCENTAGE  
OF SPORE GERMINATION BY PHYCOMYCES AT  
25° C. ON AGAR MEDIUM OF MINERALS, SUGAR,  
ASPARAGINE, AND THIAMIN

ML. OF FRACTION CA PER 25 ML. OF MEDIUM	ML. OF FRACTION DR PER 25 ML. OF MEDIUM	
	0.50	NONE
0.50.....	50.4	6.4
0.25.....	36.3	2.7
0.05.....	27.7	2.5
None.....	18.6	1.5

On the plates containing sugar, mineral salts, asparagine, and thiamin the percentage of germination was 1.5. The addition to this medium of 0.5 ml. of the Ca fraction increased the percentage to 6.4; the addition of 0.5 ml. of the Dr fraction increased it to 18.6. When the Dr and Ca fractions were both present, however, the percentage of germination was 50.4, which is greater than might be anticipated from the effects of either fraction alone. These results are illustrated in figure 1. As may be noted, the fractions of the potato extract affected not only the percentage of germination but the growth of the germ tubes.

The appearance of the plates after 69½ hours of incubation at 25° C. is shown in figure 2. The beneficial effect of the various extracts persisted and influenced the early mycelial growth.

The effect of the combined fractions on germination at 25° C. compared with their effect when used singly suggested a method of determining the presence of factor  $Z_1$  (fraction Ca) and factor  $Z_2$  (fraction Dr) in extracts of unknown composition. The method consisted of determining the effect of various amounts of the un-

known alone and in combination with the Ca and the Dr fractions prepared from potato on the germination of *Phycomyces* at 25° C. on a basic agar medium of mineral salts, sugar, asparagine, and thiamin. If the unknown and the Dr fraction combined caused a markedly greater percentage of germination than either

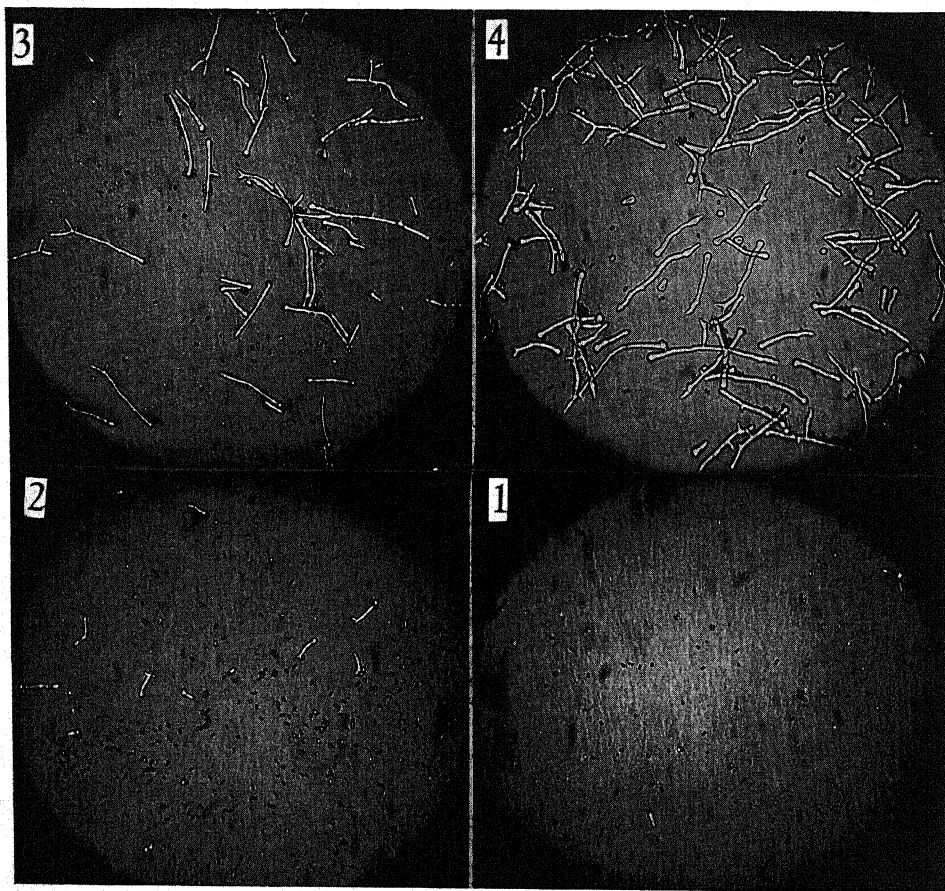


FIG. 1.—Germination of *Phycomyces* after 12 hours at 25° C. on: 1, 1 per cent agar containing sugar, mineral salts, asparagine, and thiamin; 2, same plus 0.5 ml. of Ca fraction per 25 ml. medium; 3, same plus 0.5 ml. of Dr fraction per 25 ml. medium; 4, same plus both fractions.

alone, it was considered presumptive evidence that the unknown contained effective amounts of the  $Z_1$  factor and little or no  $Z_2$ . On the other hand, if the effect of the unknown and Ca fraction combined was much more than either alone, it would be evidence for the presence of factor  $Z_2$  and little or no  $Z_1$  in the unknown. If both factors were present in effective amounts, the unknown should favor germination considerably when used alone, and addition of either the Dr or the Ca fraction should not cause marked improvement.

The early mycelial growth of *Phycomyces* in liquid culture containing sugar, mineral salts, asparagine, and thiamin was benefited by the addition of suitable amounts of the Ca fraction or of the Dr fraction. Here again the combined frac-

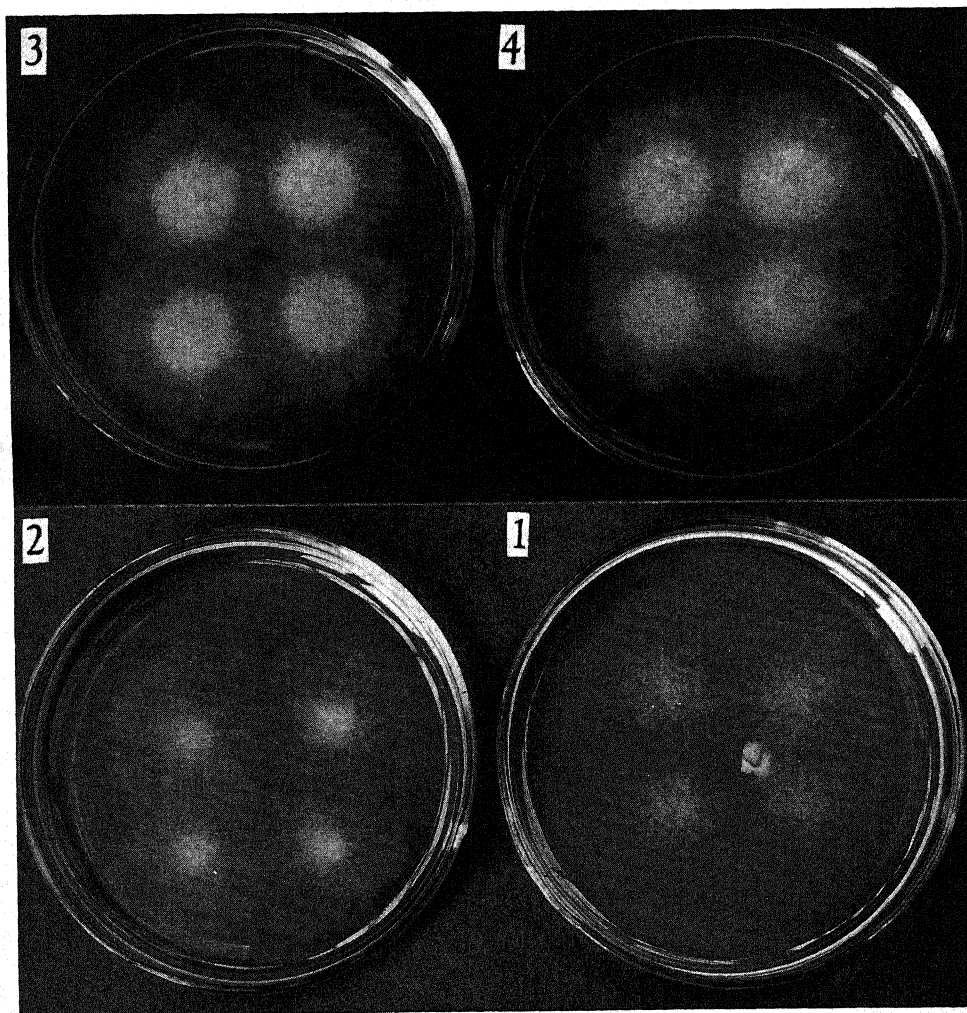


FIG. 2.—Growth of *Phycomyces* after 69½ hours at 25° C. on: 1, 1 per cent agar containing sugar, mineral salts, asparagine, and thiamin; 2, same plus 0.5 ml. of Ca fraction per 25 ml. medium; 3, same plus 0.5 ml. of Dr fraction per 25 ml. medium; 4, same plus both fractions. Each plate inoculated in four places with drop of spore suspension.

tions were much more effective than would be anticipated from their activity when used alone. This suggested another way of determining the presence of  $Z_1$  or  $Z_2$  in an unknown solution (table 2).

In this experiment a biotin concentrate from P. W. WEST containing 12.0  $\gamma$  of biotin and 3.2 mg. of solids per milliliter was used in combination with the Dr fraction in the basic medium. The Ca fraction alone, the Dr fraction, and various combinations of the two were used at the same time. In the basic medium *Phycomyces* produced in a pair of flasks 0.2 mg. of dry matter at 25° C. in 71½ hours. The addition of 0.05 ml. of fraction Ca increased the yield to 1.3 mg. and 0.5 ml. to 3.7 mg. The addition of 0.5 ml. of Dr resulted in 31.5 mg. dry weight

TABLE 2

EFFECT OF CA FRACTION AND BIOTIN CONCENTRATE IN COMBINATION WITH DR FRACTION UPON DRY WEIGHT OF PHYCOMYCES PER CULTURE PRODUCED IN 71½ HOURS AT 25° C. LARGEST AMOUNT OF BIOTIN CONCENTRATE CONTAINED 0.2  $\gamma$  BIOTIN. BASIC MEDIUM TO WHICH ADDITIONS WERE MADE CONTAINED MINERALS, SUGAR, ASPARAGINE, AND THIAMIN

ML. OF BIOTIN CONCENTRATE OR CA FRACTION ADDED PER 25 ML. OF MEDIUM	ML. DR FRACTION ADDED PER 25 ML. OF MEDIUM	
	0.50	NONE
0.166 biotin concentrate.....	27.7 mg.	.....
0.083 biotin concentrate.....	31.2	.....
0.0083 biotin concentrate.....	29.5	.....
0.00083 biotin concentrate.....	27.4	.....
0.000083 biotin concentrate.....	25.3	.....
None.....	31.5	0.2 mg.
0.50 fraction Ca.....	84.2	3.7
0.25 fraction Ca.....	68.8	2.1
0.05 fraction Ca.....	50.6	1.3

of mycelium (table 2). When the two fractions were combined the weights of the mycelium produced were 50 mg. or more. However, none of the combinations of the biotin concentrate with the Dr fraction gave yields higher than the Dr alone. It seems reasonable to conclude that the biotin concentrate in the amounts used contained little of the  $Z_1$  factor, and that biotin and the  $Z_1$  factor are not identical.

A similar procedure for determining the presence of  $Z_1$  or  $Z_2$  based on the effects on gametic reproduction at 25° C. of the Ca and Dr fractions alone and combined has also been used in some instances.

### Experimentation

PRESENCE OF FACTOR Z IN PEPTONE.—Neopeptone was found to be superior to the potato extract in stimulating germination and early mycelial growth of *Phycomyces* in media which contained thiamin. This is illustrated in the following experiments.

An extract of potato, P<sub>2</sub>, the preparation of which was described earlier (5), was

added in the amounts given in table 3 to solution 1 containing 1 per cent Difco agar and 2 gm. asparagine per liter. Neopeptone was also used in various quantities to supplement the basic medium. The germination of spores of *Phycomyces* on the basic medium and the media supplemented with potato extract or peptone was determined as already described. A comparison of the germination in the presence of the peptone with that on the media containing potato extract shows that the former was considerably more beneficial than the latter. For example, the addition of 0.0125 gm. of peptone per plate increased the percentage of germination to 73.8, while the addition of 0.015 gm. dry matter of the potato extract increased the germination to 4 per cent as compared with 0.4 per cent in the basic medium, which was supplemented with thiamin alone (table 3).

TABLE 3  
EFFECT OF POTATO EXTRACT AND PEPTONE ON GERMINATION OF  
SPORES OF PHYCOMYCES AT 25° C. ON AGAR MEDIUM OF  
MINERALS, SUGAR, ASPARAGINE, AND THIAMIN

DRY MATTER IN POTATO EXTRACT ADDED PER 25 ML. OF MEDIUM (GM.)	PERCENTAGE GERMINATION	NEOPEPTONE ADDED PER 25 ML. OF MEDIUM (GM.)	PERCENTAGE GERMINATION
0.155.....	90.0	0.25.....	85.3
0.078.....	72.5	0.10.....	93.3
0.031.....	38.5	0.05.....	91.9
0.015.....	4.0	0.025.....	82.0
0.0077.....	2.2	0.0125.....	73.8
0.0015.....	0.7	0.0025.....	8.1
0.0000.....	0.4	0.00025.....	1.1
		0.00000.....	0.4

The early growth of the mycelium also showed the greater benefit from peptone (table 4). In this instance the potato extract ( $P_2$ ) or peptone was added to 25-ml. quantities of solution 1 containing 2 gm. asparagine per liter in 125-ml. Pyrex flasks. The experiment was performed in duplicate. After 72 hours of incubation at 25° C. the dry weights of the mycelium were determined. Even 1 mg. of peptone per 25 ml. of medium increased the growth to 15.3 mg. as compared with 1.7 mg. in the solution supplemented with thiamin only. A maximum of 119.1 mg. was secured with 0.01 gm. of peptone per 25 ml. of medium. The largest amounts of peptone and of potato extract which were used decreased growth.

Judging from the effectiveness of peptone in promoting germination and early mycelial growth, it contained both the  $Z_1$  and  $Z_2$  factors. Its action in combination with the Ca or Dr fractions on early mycelial growth and on gametic reproduction was determined also. From these results, which will not be detailed here, it appeared that peptone is rich in factor  $Z_1$  and less rich in  $Z_2$ .



SUBSTITUTE FOR POTATO DEXTROSE AGAR.—Because of the favorable effect on the growth of *Phycomyces* of a medium containing thiamin and peptone, it has been used in preference to potato dextrose agar for maintaining stock cultures of filamentous fungi. Many species of *Phytophthora*, *Pythium*, *Fusarium*, and of the Mucoraceae grow well upon the thiamin-peptone medium.<sup>2</sup> It is simpler to prepare than potato-dextrose agar and has the further advantage of being transparent. No systematic attempt has been made to test groups of organisms upon it or to vary the constituents to determine their optimum proportions. It may not be so favorable for gametic reproduction as other media which can be devised, but its usefulness as a stock medium recommends it.

TABLE 4

EFFECT OF POTATO EXTRACT AND PEPTONE ON 72-HOUR GROWTH OF  
PHYCOMYCES AT 25° C. IN LIQUID MEDIUM OF MINERALS,  
SUGAR, ASPARAGINE, AND THIAMIN

DRY MATTER IN POTATO EXTRACT ADDED PER 25 ML. OF MEDIUM (GM.)	DRY WEIGHT OF MYCELIUM PER CULTURE (MG.)	NEOPEPTONE ADDED PER 25 ML. OF MEDIUM (GM.)	DRY WEIGHT OF MYCELIUM PER CULTURE (MG.)
0.31.....	87.4	0.2.....	15.0
0.124.....	129.1	0.1.....	27.3
0.062.....	113.8	0.05.....	95.1
0.031.....	88.9	0.02.....	119.1
0.0124.....	54.1	0.01.....	103.1
0.0062.....	27.8	0.005.....	57.8
0.0031.....	8.9	0.001.....	15.3
0.00062.....	2.3	0.0000.....	1.7
0.00000.....	1.7		

PRESENCE OF Z FACTORS IN AGAR.—The presence of the Z factor in agar has been previously reported (2). It was found possible to remove the Z factor in part by extracting the agar with methyl alcohol or aqueous pyridine. The effect of an agar extract upon the early growth of *Phycomyces* in a solution supplemented with thiamin is illustrated by the following experiments.

The agar extract was prepared<sup>3</sup> as follows: 424 gm. of Difco agar was treated in a percolator with 4 liters of 5 per cent pyridine, and the agar then washed with 2 liters of water and 4 liters of methyl alcohol. The brownish colored filtrates were combined, evaporated to small volume, and enough methyl alcohol added to make approximately 66 per cent. The precipitate was removed and the liquid again evaporated to small volume and treated with alcohol as before. The alcoholic liquor was evaporated to small volume and made up with distilled water to 200 ml.

<sup>2</sup> This medium contained per liter: 50 gm. dextrose, 2 gm. asparagine, 1.5 gm.  $\text{KH}_2\text{PO}_4$ , 0.5 gm.  $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$ , 0.1 mg. thiamin, 0.4 gm. peptone, and 1 per cent agar.

<sup>3</sup> The assistance of FREDERICK KAVANAGH in preparing this extract is acknowledged.

It contained 6.53 per cent solids, and 1 ml. represented the extract from 2.15 gm. of air-dry agar.

EFFECT OF AGAR EXTRACT ON GROWTH.—Various quantities of the agar extract were added to 25 ml. of solution 1 containing 2 gm. asparagine per liter or to the same solution without asparagine. The liquid was inoculated with a spore suspension of *Phycomyces* and incubated at 25° C. Dry weights were determined at the end of 72 hours (table 5). The experiment was performed in duplicate. Only a trace of mycelium had developed in 72 hours in the solution with no agar extract. The addition of 0.1 ml. of agar extract containing 6.53 mg. of solids resulted

TABLE 5  
EFFECT OF AGAR EXTRACT ON 72-HOUR GROWTH OF  
PHYCOMYCES AT 25° C. IN MEDIUM OF MINERAL  
SALTS, SUGAR, AND THIAMIN WITH 2 GM. ASPARA-  
GINE PER LITER OR WITH NO ASPARAGINE

ML. OF AGAR EXTRACT ADDED TO 25 ML. OF MEDIUM	DRY WEIGHT OF MYCELIUM PER CULTURE (MG.)	
	MEDIUM WITH ASPARGINE	MEDIUM WITH NO ASPARGINE
None.....	Trace	.....
0.1.....	5.0	.....
0.2.....	15.0	.....
0.4.....	15.6	.....
0.8.....	15.5	.....
1.2.....	20.9	3.1
1.6.....	20.9	3.6
2.0.....	14.4	4.0

in the production of 5 mg. of dry mycelium. Larger amounts of agar extract were more beneficial, the maximum benefit being secured with 1.2 or 1.6 ml. of extract per 25 ml. of medium, 2 ml. resulting in less growth than was obtained with a smaller amount. The growth in a medium containing agar extract but no asparagine indicated that the amount of available nitrogen in the agar extract was small. Because of the beneficial effect of the extract on the early mycelial growth at 25° C. in a basic medium of sugar, minerals, asparagine, and thiamin, it was concluded that the agar extract contained factor Z.

Although it appeared from this experiment that the agar extract contained factor Z, it seemed desirable to determine whether its beneficial action affected more than the early mycelial growth and what effect certain changes in the composition of the medium might have. Time curves were determined for the growth of *Phycomyces* in solution 1 with 5 gm. of asparagine per liter, in the same solution plus 1 ml. of agar extract per flask (25 ml. of medium), in the same solution plus 0.1 ml.

of agar extract, in solution 2 with 10 gm. of asparagine per liter, and in solution 2 plus 1 ml. of agar extract per flask. At the same time a few determinations were made of growth in solution 1, containing agar extract but no asparagine, no sugar, or no thiamin. The experiment was performed in duplicate and growth was measured by dry weights at 24-hour intervals (table 6; fig. 3). The beneficial effect of 1 ml. of agar extract was still evident after  $8\frac{1}{2}$  days of growth. With the agar extract growth increased to a maximum of 416 mg. at  $6\frac{1}{2}$  days and then decreased.

TABLE 6

EFFECT OF AGAR EXTRACT ON GROWTH OF PHYCOMYCES FOR VARIOUS PERIODS OF TIME IN SOLUTIONS OF MINERAL SALTS, SUGAR, ASPARAGINE, AND THIAMIN. SALT CONCENTRATION OF SOLUTION 2 WAS 10 TIMES THAT IN SOLUTION 1

PERIOD OF GROWTH IN DAYS	DRY WEIGHT OF MYCELIUM PER CULTURE (MG.)							
	SOLUTION 1 WITH 5 GM. ASPARAGINE PER LITER	PLUS 1 ML. AGAR EXTRACT PER 25 ML. MEDIUM	PLUS 0.1 ML. AGAR EXTRACT PER 25 ML. MEDIUM	SOLUTION 1 WITH NO ASPARAGINE BUT 1 ML. AGAR EXTRACT PER 25 ML. MEDIUM	SOLUTION 1 WITH NO SUGAR BUT 1 ML. AGAR EXTRACT PER 25 ML. MEDIUM	SOLUTION 1 WITH NO THIAMIN BUT 1 ML. AGAR EXTRACT PER 25 ML. MEDIUM	SOLUTION 2 WITH 10 GM. ASPARAGINE PER LITER	PLUS 1 ML. AGAR EXTRACT PER 25 ML. MEDIUM
$1\frac{1}{2}$ .....	0.0	1.6	0.0	.....	.....	.....	0.7	1.9
$2\frac{1}{2}$ .....	0.5	8.5	3.9	.....	.....	.....	1.0	4.7
$3\frac{1}{2}$ .....	14.3	63.1	28.0	.....	.....	.....	1.6	10.6
$4\frac{1}{2}$ .....	141.3	228.1	206.3	.....	.....	.....	27.2	51.3
$5\frac{1}{2}$ .....	260.3	391.2	345.1	.....	.....	.....	304.2	330.4
$6\frac{1}{2}$ .....	292.6	416.1	363.6	.....	.....	.....	482.5	627.0
$7\frac{1}{2}$ .....	300.1	391.2	365.0	7.4	1.2	14.7	415.1	711.6
$8\frac{1}{2}$ .....	327.0	363.7	363.4	.....	.....	.....	621.8	734.4
$9\frac{1}{2}$ .....	346.7	330.5	359.6	8.2	1.3	16.4	498.6	734.9
$10\frac{1}{2}$ .....	320.6	318.2	359.5	.....	.....	.....	640.0	714.3
$11\frac{1}{2}$ .....	349.0	317.9	364.4	.....	.....	.....	688.3	741.2
$12\frac{1}{2}$ .....	.....	342.7	362.0	.....	.....	.....	676.2	693.3
$13\frac{1}{2}$ .....	.....	322.8	363.3	11.0	1.0	20.2	649.8	655.0

Without the agar extract growth increased for  $9\frac{1}{2}$  days until at that time growth in the medium with and without the agar extract was approximately the same. The 0.1-ml. agar extract was somewhat less effective than the 1-ml. extract.

Growth was less rapid for the first  $4\frac{1}{2}$  days in solution 2 than in solution 1. Thereafter, however, the dry weight of the mycelium in solution 2 was considerably in excess of that in solution 1. The maximum dry weight (688.3 mg.) in solution 2 was attained in  $11\frac{1}{2}$  days. I have no explanation for the irregularities in growth in solution 2 after  $6\frac{1}{2}$  days (fig. 3). Growth in solution 2 containing agar extract was superior to growth in the same solution without extract for the entire period of the experiment. The agar extract contained approximately 0.2 millimicromole of thiamin per milliliter but very little available carbon or nitrogen.

In this experiment approximately half as much growth was obtained in solution 1 as in solution 2. It is not possible to say whether the increased buffer action re-

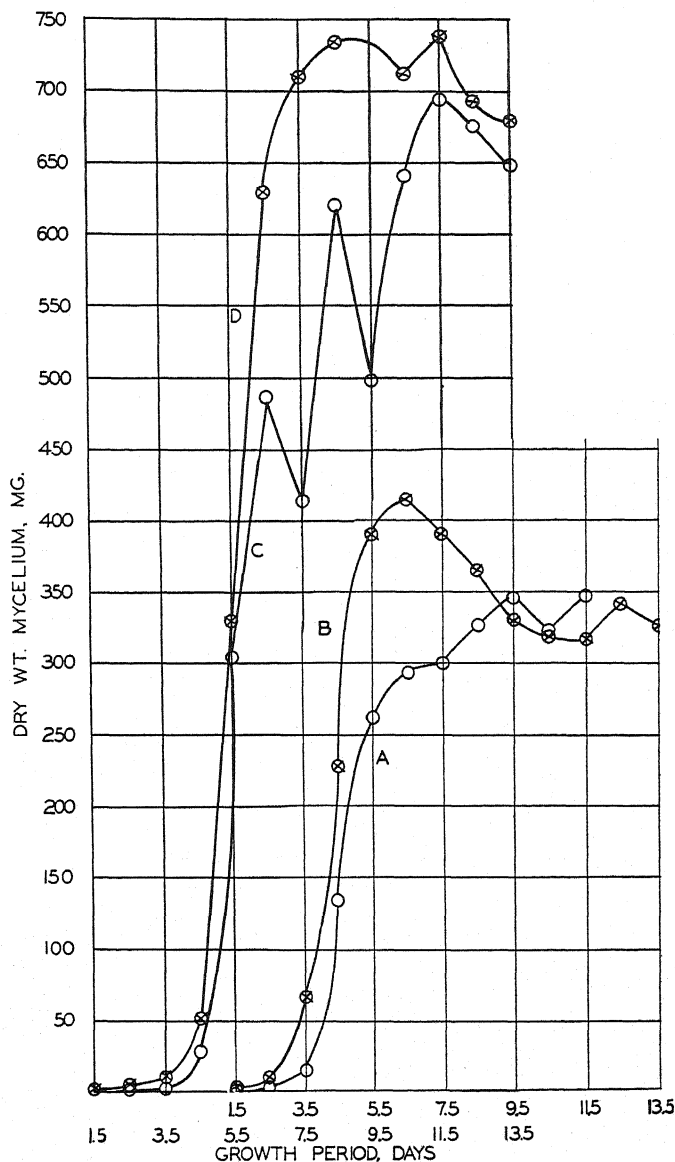


FIG. 3.—Time curves for growth of *Phycomyces*: A, in solution 1; B, in same solution plus 1 ml. agar extract per 25 ml. medium (both solutions contained 5 gm. asparagine per liter and excess thiamin); C, in solution 2; D, in solution 2 plus 1 ml. agar extract per 25 ml. medium (both solutions contained 10 gm. asparagine per liter and excess thiamin).

sulting from the greater quantity of phosphate, the greater quantity of minerals supplied, the greater amount of asparagine in solution 2, or some other factor, was responsible for this difference in total growth. It is probable that growth in solution 1 was limited by the amount of asparagine supplied. If this assumption is correct, then the beneficial action of the agar extract on growth in this solution was the result of some constituent of the agar other than thiamin, available nitrogen, or available carbon. The same statement would apply to the beneficial effect of agar extract on growth in solution 2.

In any event it seems probable that the beneficial effect of the agar extract is not because of its mineral content, since it was effective in both the weaker and stronger salt solution. It is possible that factor Z may beneficially influence the mature dry weight of *Phycomyces* at 25° C. as well as early mycelial growth, provided other factors (for example—thiamin, the nitrogen supply, sugar, or mineral salts) are not limiting. To determine this with certainty will probably not be possible until the constituents of factor Z are available in pure form.

In the experiment just described thiamin was supplied in *luxus* amounts. What effect would agar extract have on growth in solutions where thiamin is the limiting factor? This was determined by growing *Phycomyces* for 12 days in solution 2 with 10 gm. of asparagine per liter and various amounts of thiamin and in the same solutions plus 1 ml. of agar extract per 25 ml. of medium. The experiment was performed in duplicate. Figure 4 shows that the agar extract contained per milliliter between 0.1 and 0.2 millimicromoles of thiamin or its intermediates. The greater growth in the medium containing the agar extract and 0.1 or 0.2 millimicromoles of thiamin might be accounted for by the thiamin added. For amounts of added thiamin between 0.4 to 1.6 millimicromoles the differences in growth in the media with and without agar extract appear to be greater than can be accounted for by the thiamin content of the extract. Whether factor Z affects the dry weight of the mature mycelium of *Phycomyces* when thiamin is present in limited quantity is uncertain. Further investigation of this question should probably be postponed until the constituents of factor Z are available in pure form.

Each of the curves in figure 4 consists of two parts, one for the smaller amounts of thiamin and one for the larger amounts. Since little surface mycelium was formed in the solutions containing up to 0.4 millimicromoles of thiamin as compared with considerable surface and aerial mycelium with amounts of 0.6 millimicromoles thiamin and greater, the two parts of each curve may be related to this difference in growth habit.

FACTOR Z<sub>1</sub> IN AGAR EXTRACT.—The effect of the Ca and Dr fractions from potato on the action of agar extract was determined. It appeared that the agar extract contained considerable factor Z<sub>1</sub> and little Z<sub>2</sub>. The addition of 0.5 ml. of the

Dr fraction to 25 ml. of medium produced 25.6 mg. of mycelium as compared with 2 mg. in the check solutions and 8.8 mg. in the solutions plus 1 ml. of agar extract (table 7). When 1 ml. of agar extract and 0.5 ml. of the Dr fraction were both present 76.2 mg. of mycelium was produced.

CATHODE AND ANODE LIQUORS FROM ELECTRODIALYZED AGAR.—Through the courtesy of C. E. MARSHALL, the cathode and anode liquors from a 30-gm. sample of shredded agar were obtained. Each of these liquors was neutralized and evaporated under reduced pressure to 100 ml. The concentrated anode liquor contained

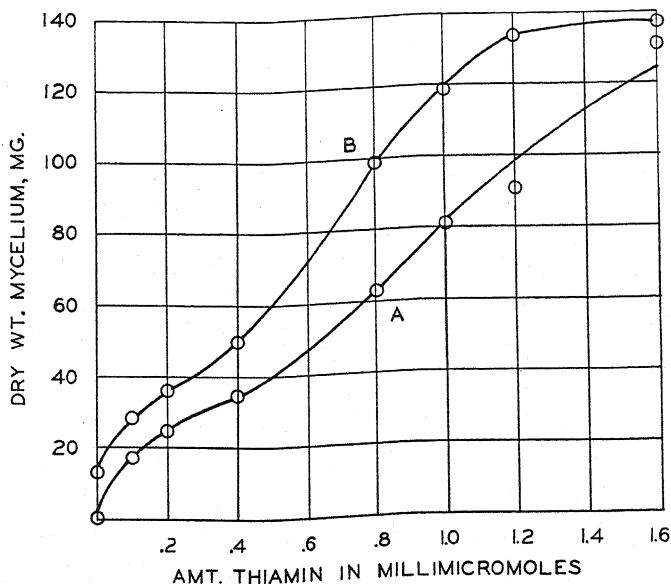


FIG. 4.—Growth of *Phycomyces* in solution 2 with 10 gm. asparagine per liter and various amounts of thiamin per flask: A, without agar extract; B, with 1 ml. agar extract per 25 ml. medium.

3.82 per cent dry matter; the cathode liquor, 1 per cent. Heated at 75° C., the ash in the anode liquor was 2.5 per cent, volatile dry matter 1.32 per cent; in the cathode liquor the ash was 0.27 per cent, volatile dry matter 0.73 per cent.

The effect of these liquors upon the early mycelial growth of *Phycomyces* was determined by addition of various quantities of each to solution 1 containing 2 gm. asparagine per liter. It was found (table 8) that the cathode liquor was beneficial and the anode liquor ineffective or somewhat toxic. The cathode liquor therefore contained appreciable amounts of factor Z, while the anode liquor contained little or none; in fact, a beneficial effect was obtained with 0.5 ml. of the cathode liquor which contained 5 mg. of dry matter. On a dry-matter basis the cathode liquor was about as effective as the agar extract previously used, but less effective than potato extract or neopeptone.

FACTOR  $Z_1$  IN CATHODE LIQUOR.—When the cathode and anode liquors were used together as supplements to solution 1, the growth was intermediate between that obtained with the anode liquor or cathode liquor alone. When the anode liquor was combined with the Dr or Ca fraction from potato, no increase in growth over that with the liquor alone or the fractions alone was obtained. The cathode liquor in combination with the Ca fraction gave no increase, but with the Dr fraction the growth was much greater than with the cathode liquor or the Dr fraction alone. These results were taken to mean that the cathode liquor contained effective amounts of the  $Z_1$  factor but little or no  $Z_2$ , and that the anode liquor lacked both.

TABLE 7

EFFECT OF CA AND DR FRACTIONS ON 70-HOUR GROWTH OF PHYCOMYCES AT 25° C. IN SOLUTION 1 CONTAINING 2 GM. ASPARAGINE PER LITER AND AGAR EXTRACT

ML. AGAR EXTRACT ADDED PER 25 ML. OF MEDIUM	DRY WEIGHT OF MYCELIUM PER CULTURE (MG.)			
	NO ADDITION EXCEPT AGAR EXTRACT	0.5 ML. DR FRACTION ADDED PER 25 ML. OF MEDIUM	0.5 ML. CA FRACTION ADDED PER 25 ML. OF MEDIUM	0.5 ML. CA FRACTION AND 0.5 ML. DR FRACTION ADDED PER 25 ML. OF MEDIUM
0.0.....	2.0	25.6	4.2	81.6
0.25.....	5.3	66.3	7.7	.....
1.0.....	8.8	76.2	9.7	.....

BIOTIN AND FACTOR  $Z_1$ .—ROBBINS and HAMNER (5) suggested that factor  $Z_1$  might be biotin. The Ca fraction from potato, the anti gray-hair factor concentrate from MORGAN, and peptone—all of which contain factor  $Z_1$  contain biotin also. By the use of *Ashbya gossypii* (6) I found per gram of dry matter in the Ca fraction of potato  $1800 \times 10^{-3} \gamma$  of biotin, in neopeptone 2100, in the anti gray factor concentrate 900, and in the Dr fraction from potato none. It appears, however, that the effective material in the Ca fraction of potato is not biotin.

Two biotin concentrates<sup>4</sup> were tested. The first was stated by WEST to contain 1.2  $\gamma$  of biotin per milliliter and to have 0.3 mg. of dry matter per milliliter; the second was described earlier in this paper. Neither concentrate had much effect on spore germination and early mycelial growth of *Phycomyces* in solution 1. Neither concentrate affected the action of the Dr fraction from potato on the spore germination or early mycelial growth. From 0.00001  $\gamma$  to 0.2  $\gamma$  of biotin was used per flask containing 25 ml. of medium.

<sup>4</sup> I am indebted to Dr. P. W. WEST for these concentrates.

The coenzyme R concentrate was kindly furnished by F. E. ALLISON. Coenzyme R has been stated to be identical with biotin (1, 7). In contrast to the biotin concentrate from WEST, the concentrate of coenzyme R had a distinctly beneficial effect upon the early mycelial growth of *Phycomyces* in solution 1. The coenzyme R concentrate and the Dr fraction of potato combined were more beneficial than either alone. It was concluded that the coenzyme R concentrate contained some of the  $Z_1$  factor.

TABLE 8

EFFECT OF CATHODE AND ANODE LIQUORS FROM AGAR ALONE AND IN PRESENCE OF EACH OTHER, THE CA OR DR FRACTIONS, ON 72-HOUR GROWTH OF PHYCOMYCES AT 25° C. IN SOLUTION OF MINERALS, SUGAR, ASPARAGINE, AND THIAMIN

ML. OF CATHODE OR ANODE LIQUOR ADDED PER 25 ML. OF MEDIUM	DRY WEIGHT OF MYCELIUM PER CULTURE (MG.)						
	CATHODE LIQUOR ADDED	ANODE LIQUOR ADDED	CATHODE AND ANODE LIQUOR ADDED	CATHODE LIQUOR AND 0.5 ML. DR FRACTION ADDED	CATHODE LIQUOR AND 0.5 ML. CA FRACTION ADDED	ANODE LIQUOR AND 0.5 ML. DR FRACTION ADDED	ANODE LIQUOR AND 0.5 ML. CA FRACTION ADDED
0.....	2.0	2.0	2.0	38.7	7.3	38.7	7.3
0.1.....	1.8	1.9	3.0	.....	.....	.....	.....
0.5.....	4.7	0.3	4.5	.....	.....	.....	.....
1.0.....	5.0	0.5	5.0	64.7	8.5	11.4	5.8
2.0.....	8.5	2.4	4.7	.....	.....	.....	.....

PANTOTHENIC ACID AND FACTOR  $Z_1$ .—The effect of pure synthetic calcium pantothenate<sup>5</sup> on the 72-hour growth of *Phycomyces* was determined in solution 1 with 2 gm. of asparagine per liter. Calcium pantothenate in amounts ranging from 0.01 to 10  $\gamma$  per flask was used alone and in combination with the Ca and Dr fractions from potato. The calcium pantothenate was ineffective, and it was concluded that pantothenic acid is not identical with the  $Z_1$  or  $Z_2$  factors.

GLUTAMIN OR PARA-AMINO BENZOIC ACID AND FACTOR  $Z_2$ .—The effect of glutamin and of para-amino benzoic acid<sup>6</sup> on the 72-hour growth of *Phycomyces* was determined in solution 1 with 2 gm. asparagine per liter. Each compound was tested in amounts ranging from 0.001 to 5.0 mg. per flask and in combination with various amounts of the Ca and Dr fractions from potato. Neither compound appeared to be factor  $Z_1$  or factor  $Z_2$ .

### Discussion

The experimental results reported here support the conclusion of ROBBINS and of ROBBINS and HAMNER that unidentified growth factors influence the develop-

<sup>5</sup> Merck and Co. supplied this material.

<sup>6</sup> These preparations were kindly supplied by Dr. PAUL GYORGY.



ment of *Phycomyces* in a medium supplemented with thiamin. The results show further that a pyridine extract of agar and the cathode liquor from electro dialyzed agar contain appreciable quantities of factor  $Z_1$  and little or no  $Z_2$ . Peptone was found to be rich in factor  $Z_1$  and less rich in  $Z_2$ .

It is not possible as yet to identify either factor.  $Z_1$  seems to belong in the vitamin B complex. It is probably not identical with biotin, pantothenic acid, vitamin B<sub>2</sub>, pyridoxine (vitamin B<sub>6</sub>), or thiamin. It is soluble in water and in aqueous alcohol or acetone; it is thermostable and migrates to the cathode in electrodialysis. It is strongly adsorbed by charcoal, from which it may be eluted by ammoniacal acetone. From evidence which will be presented elsewhere, one or both of these factors seem important for the development of other plants also. Since some of the growth substances of this general character (for example—pantothenic acid, thiamin, and pyridoxine) have been found necessary for both plants and animals, it is probable that factor  $Z_1$  is a vitamin and may represent an addition to those already known.

As knowledge of the relation of vitamins and vitamin-like substances to plants increases, it seems evident that the relation is complex. Many such substances are required in the metabolism of a plant, just as they are in that of an animal, and the ability of a plant to synthesize one or more of them in sufficient amounts may determine the rapidity and completeness of its development. *Phycomyces* is unable to synthesize thiamin from the sugar, sulphate, and asparagine in the basic medium; unless supplied with that vitamin or its intermediates it is unable to grow. In the presence of thiamin, however, its development is influenced by its ability to make adequate quantities of two unidentified factors,  $Z_1$  and  $Z_2$ . Important as thiamin is for the growth of *Phycomyces*, it is only one of many substances of similar function necessary for maximum and complete development. Most of these are synthesized in sufficient amounts by the organism. It is interesting to speculate on what the limiting factors for growth might be if all the vitamins and similar growth substances, as well as nutrients, foods, water, and oxygen, were supplied in luxur amounts.

### Summary

1. Methods of estimating factors  $Z_1$  and  $Z_2$  in solutions of unknown composition are described.
2. Neopeptone was found to be rich in  $Z_1$  and less rich in  $Z_2$ .
3. A pyridine extract of Difco agar was found to contain factor  $Z_1$  but little  $Z_2$ . This extract influenced the mature dry weight of *Phycomyces* in the presence of excess thiamin.
4. The cathode liquor from shredded agar contained  $Z_1$  but little or no  $Z_2$ . The anode liquor contained neither.

5.  $Z_1$  was not found to be identical with biotin, pantothenic acid, glutamin, or para-amino benzoic acid.  $Z_2$  was not found to be identical with glutamin or para-amino benzoic acid.

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# INFLUENCE OF SULPHUR DEFICIENCY ON METABOLISM OF THE SUNFLOWER

CONTRIBUTIONS FROM THE HULL BOTANICAL LABORATORY 520

SCOTT V. EATON

(WITH ONE FIGURE)

## Introduction

There are few detailed studies of the effects of sulphur deficiency on the metabolism of plants. NIGHTINGALE (21) studied the effects in the tomato. The minus-sulphur plants were yellowish green in color and weighed much less than the plus-sulphur plants, although they were as tall as, or taller than, the latter. He explained this growth in height by the rapid proteolysis that was evidently going on in the sulphur-deficient plants. His analyses showed that these plants were high in soluble organic sulphur and nitrogenous compounds in comparison with the plants grown in a complete nutrient solution. NIGHTINGALE recognized that this proteolysis was abnormal, in that it took place in plants that were much higher in carbohydrates than were the plus-sulphur plants. Usually proteolysis is associated with a reduction in carbohydrates.

The situation in the soybean (5) was similar to that in the tomato, in that proteolysis and reutilization of the resulting compounds seemed to account for the elongation of the minus-sulphur plants. The proteolysis, however, was more in accord with that usually going on, for while the sulphur-deficient plants were high-carbohydrate, if all forms of carbohydrates were considered, they were much lower in the more available carbohydrates, reducing sugars and sucrose, than were the plus-sulphur plants.

The main object of the present work was to secure further data on the effects of sulphur deficiencies on metabolism, especially in regard to this theory of reutilization. Stem elongation of the minus-sulphur soybean plants was accounted for by proteolysis and the translocation of the resulting nitrogenous and sulphur compounds to the tips of the plants. But whole stems were analyzed in the case of the soybean. This was also true for the most part of NIGHTINGALE's experiments with the tomato, although analyses were made for some of the sulphur fractions at different levels of the plant. In the present study the stem was analyzed at three levels, with the thought that if proteins of lower levels were being hydrolyzed and the resulting materials translocated to the tip, then positive gradients from below upward of the nitrogenous and sulphur fractions might be found.

No attempt is made to review the literature on sulphur. The literature reviews of the papers just referred to are adequate. McMURTREY (17) has reviewed the literature on sulphur, and WILLIS (35) has an extensive list of references with abstracts.

### Material and methods

PRELIMINARY WORK.—Sulphur-deficient sunflower plants were grown for the first time in the spring of 1934, in connection with the work on the soybean (5). Decided symptoms of deficiency were obtained. The weights of tops and roots were taken and the top-root ratios calculated (5). No analyses were made.

During the spring of 1935, sunflower plants were grown according to the triangular method (11) to determine the best concentrations of the salts of the nutrient solution for growth of this plant. Some of them developed decided boron-deficiency symptoms, so in the autumn of 1935 and the spring of 1936 experiments were conducted to determine the best concentration of boron for the growth of the sunflower. The concentrations were extended into the toxic realm. The data have been reported (6).

TABLE 1  
PARTIAL VOLUME MOLECULAR CONCENTRATION  
OF SALTS OF NUTRIENT SOLUTION

SULPHUR	$\text{KH}_2\text{PO}_4$	$\text{Ca}(\text{NO}_3)_2$	$\text{MgSO}_4$	$\text{MgCl}_2$
Minus.....	0.0045	0.0090	.....	0.0045
Plus.....	0.0045	0.0090	0.0045	.....

FINAL PLAN.—Sunflower plants (*Helianthus annuus* L., variety Mammoth Russian) were grown from seed in pure quartz sand that had been washed thoroughly with distilled water. The plan of growing the plants was essentially the same as previously described (5), except that in the present work the natural day length was not supplemented by artificial light.

The preliminary work had shown that solution IR<sub>2</sub>S<sub>4</sub> of the triangle (11) was best for growth of the sunflower. Table 1 gives its composition. Each solution has an osmotic pressure of one atmosphere. Boric acid at a concentration of 0.5 p.p.m. of boron was kept constantly in the nutrient solution, this being the concentration which the preliminary work showed was optimal. During the last 10 days of the experiment the concentration was increased to 0.75 p.p.m. Enough manganese chloride to give a concentration of 0.25 p.p.m. of manganese was also added.

The nutrient solution was applied in excess to the sand daily. The pots were flushed thoroughly twice a week with distilled water, and to the water was added iron as ferric chloride at a concentration of about 1 p.p.m. of iron.

The seed was planted April 4, 1939, and the plants harvested May 17. The plants were put in the darkroom the evening before and left until 8:00 A.M. the next morning, when the sampling was begun. At the time of harvest a few of the plants had begun to form heads; none of these were included in the samples. A number of the plus- and minus-sulphur plants were grown to maturity for data on the effects of sulphur deficiency on flower and seed production. These data are not reported in the present paper.

#### CHEMICAL METHODS

Fifty-three plus-sulphur and 135 minus-sulphur plants were taken for analysis. Only the stems were analyzed. In the case of soybean (5), sulphur deficiency affected the chemical composition of the stems much more than the other parts of the plant. In the present experiments each stem was divided into three sections of equal length, each section was cut into small pieces, and duplicate 100-gm. samples were taken of each.

Seventy per cent alcohol was used for the extraction instead of 80 per cent as in the previous work (5), to secure more complete extraction of the nitrogenous fractions. (STUART (28) found that, compared with water, 80 per cent alcohol gave very incomplete extraction of these compounds.) The tissue was covered with the alcohol, the alcohol heated to boiling on the steam bath, allowed to cool to room temperature, and filtered. Eight extractions were made, since tests with alpha naphthol showed that all the sugar was removed by this number of extractions.

**CARBOHYDRATE FRACTIONS.**—The carbohydrates were determined by essentially the same methods as previously described (5). In the permanganate titration the indicator ortho-phenanthroline was used (29). This decidedly increases the sharpness of the end point. Sucrose was determined after hydrolysis with invertase, as described by LOOMIS and SHULL (12). Their methods were also followed with slight modifications in the determination of starch and acid-hydrolyzable carbohydrates.

**NITROGEN FRACTIONS.**—The chief modification of the methods used previously (5) in the determination of the nitrogen fractions was that the ammonia was determined by the vacuum distillation method of VAN SLYKE (30). STUART (28) found that vacuum distillation was an efficient method of reducing the errors in the determination of amino nitrogen by the VAN SLYKE method (31). The presence of ammonia in the extract constitutes one of these errors. After the removal of ammonia by vacuum distillation, amino nitrogen was determined by the VAN SLYKE method; and as before, PHILLIPS' methods (23) were used in the determination of nitrates and amides.

Considerable difficulty was experienced in getting close duplicates in the nitrate determinations by PHILLIPS' original method, and the concentrations at the three

levels showed no regular trend. Possibly the difficulty might be accounted for by the splitting off of ammonia from organic nitrogenous compounds owing to the rather strong alkalinity of the extract. SESSIONS and SHIVE (27) got indications that even eighth-normal sodium hydroxide had a slight hydrolyzing effect on such compounds. Following PHILLIPS' (24) later suggestion, therefore, the nitrates were determined in a solution made tenth-normal with sodium hydroxide. After the acid digestion of the amides, the solution was nearly neutralized with 50 per cent sodium hydroxide, cooled, a slight excess of the sodium hydroxide added, and the amide nitrogen aerated off. No potassium carbonate was used. The solution left in the test tube was transferred to a beaker, neutralized with 1:1 sulphuric acid, and enough 50 per cent sodium hydroxide added to make 300 ml. tenth-normal. The solution was then transferred to a 800-ml. Kjeldahl flask, made up to a volume of 300 ml., and the determination completed in the regular way. All the amide nitrogen data reported in this paper, however, were obtained by PHILLIPS' original method.

**SULPHUR FRACTIONS.**—Since the total sulphur in the alcoholic residue was determined after drying to constant weight in the vacuum oven, it was deemed essential to determine whether the sunflower stems contained any volatile sulphur that would be lost during the drying process. Tests by the method of PLATENIUS (25) showed little, if any, present; so this fraction was not estimated. Total sulphur in the extract after evaporating off the alcohol was determined by the official sodium peroxide method (1), and in the residue by the Parr Co. bomb method essentially as described in their direction booklet. This bomb method is a great improvement over the open-crucible sodium peroxide method.

### Results

Symptoms of sulphur deficiency developed quickly, appearing first in the first true leaves. In less than 2 weeks after planting, these leaves were decidedly less green than were the corresponding leaves of the plus-sulphur plants. Also at this time some of the minus-sulphur plants were not so tall as the plants supplied with a complete nutrient solution. These symptoms increased in intensity as the plants became older. At the time of harvest the leaves of the sulphur-deficient plants were yellow-green in color, smaller, and the stems much thinner than in the case of the plus-sulphur plants. No definite gradation of greenness was noticed at any time. While the minus-sulphur plants were shorter than those supplied with sulphur, yet they elongated markedly. A few plants of each group were grown to maturity. Some of the sulphur-deficient plants reached a height of almost 200 cm.; the tallest plus-sulphur plant was about 300 cm. in height.

It is evident from table 2 that the stems of the minus-sulphur plants are much smaller than those of the plus-sulphur, the latter at each level weighing more than

three times the former. In each case there is an increase in green weight from the top to the bottom of the plant. The two groups do not differ much in moisture content at each level. The percentage moisture of each group decreases from the top to the bottom of the plant, as would be expected; the percentage dry weight of course shows the reverse gradation. Tables 3-8 give the data for the chemical com-

TABLE 2  
DATA FROM 100 PLANTS

STEM LEVEL	GREEN WEIGHT		PERCENTAGE WATER		PERCENTAGE DRY WEIGHT	
	MINUS S	PLUS S	MINUS S	PLUS S	MINUS S	PLUS S
Upper.....	387.7	1399.8	93.767	93.530	6.233	6.470
Middle.....	488.5	1769.6	93.143	93.147	6.857	6.853
Lower.....	628.2	2185.2	89.583	90.383	10.417	9.617

TABLE 3  
NITROGEN FRACTIONS (PERCENTAGE ON DRY-WEIGHT BASIS)

STEM LEVEL	MINUS S	PLUS S	MINUS S	PLUS S	MINUS S	PLUS S	MINUS S	PLUS S
	TOTAL N		TOTAL ORGANIC N		70% ALCOHOL-SOLUBLE N		70% ALCOHOL-INSOLUBLE N	
Upper.....	5.526	3.824	3.188	1.717	4.537	2.656	0.989	1.168
Middle.....	4.673	2.968	2.431	1.112	4.145	2.221	0.528	0.747
Lower.....	2.397	1.801	1.231	0.766	1.971	1.249	0.426	0.552
	AMMONIA N		AMINO N		AMIDE N		NITRATE N	
Upper.....	0.163	0.022	0.798	0.185	0.192	0.004	2.339	2.107
Middle.....	0.092	0.011	0.625	0.115	0.156	0.004	2.243	1.856
Lower.....	0.035	0.002	0.233	0.085	0.071	0.000	1.166	1.035

position, figured on the basis of the dry weight and also calculated on the absolute basis.

NITROGEN FRACTIONS.—Table 3 shows that there is a positive gradient from top to bottom of the stems of each group of plants, but at each level the percentage is much higher for the minus-sulphur stems than for the plus-sulphur, except of course in the case of 70 per cent alcohol-insoluble nitrogen. A comparison of the two groups at the three levels shows that the sulphur-deficient stems contain seven to seventeen times as much ammonia and two to five times as much amino nitro-

gen as the plus-sulphur. Very little amide was found in the latter stems (none at the lower level), while considerable is found in the former. Nitrates accumulate to a certain extent in the minus-sulphur stems. In each group the nitrates make up a rather large proportion of the nitrogen that dissolves in 70 per cent alcohol. Most of the total nitrogen dissolves in 70 per cent alcohol; there is a rather small amount left in the residue (70 per cent alcohol-insoluble nitrogen).

Table 4 gives the data for the nitrogen fractions on an absolute basis. The gradients of the percentage data are more or less leveled out when the data are figured on an absolute basis, because the gradients of the percentages and the absolute

TABLE 4  
NITROGEN FRACTIONS (GRAMS PER 100 PLANTS)

STEM LEVEL	MINUS S	PLUS S	MINUS S	PLUS S	MINUS S	PLUS S	MINUS S	PLUS S
	TOTAL N		TOTAL ORGANIC N		70% ALCOHOL-SOLUBLE N		70% ALCOHOL-INSOLUBLE N	
Upper.....	1.335	3.463	0.770	1.555	1.006	2.405	0.239	1.057
Middle.....	1.505	3.599	0.814	1.385	1.388	2.693	0.176	0.905
Lower.....	1.568	3.784	0.805	1.609	1.290	2.624	0.278	1.160
	AMMONIA N		AMINO N		AMIDE N		NITRATE N	
Upper.....	0.039	0.019	0.192	0.167	0.046	0.003	0.565	1.908
Middle.....	0.030	0.013	0.209	0.139	0.052	0.004	0.751	2.250
Lower.....	0.022	0.004	0.145	0.178	0.046	0.000	0.763	2.175

weights of the stem sections are in opposite directions (table 2). Since the plus-sulphur stems weighed much more than the minus-sulphur, they are higher in the various nitrogen fractions, except ammonia, amide, and amino acids. The fact that despite the much smaller stems a larger amount of these fractions is present in the sulphur-deficient stems emphasizes their accumulation as a result of sulphur deficiency.

CARBOHYDRATE FRACTIONS.—Table 5 records the data for the carbohydrate fractions on a percentage basis. The minus-sulphur stems are somewhat higher in starch and acid-hydrolyzable carbohydrates but are much lower in reducing sugars and sucrose than the plus-sulphur. They contain less than one-fourth as much reducing sugar and less than one-third as much sucrose as the stems of the plants grown with complete nutrient. A large amount of reducing sugar is found in the plus-sulphur stems, reaching at the upper level more than 19 per cent of the dry weight. Reducing sugars make up a rather large part of the total carbohydrates of



both groups of plants; relatively small amounts of polysaccharides and sucrose are present. The percentages of total carbohydrates are not given, but it is evident that, because of the large sugar content of the plus-sulphur stems, they are to be considered high-carbohydrate in comparison with the minus-sulphur.

The gradient of reducing sugar is positive downward in each group of plants, but the data for sucrose exhibit no definite trend. While the starch gradient is positive downward, the acid-hydrolyzable gradient is in the opposite direction.

TABLE 5  
CARBOHYDRATE FRACTIONS (PERCENTAGE ON DRY-WEIGHT BASIS)

STEM LEVEL	REDUCING SUGARS		SUCROSE		STARCH		ACID-HYDROLYZABLE CARBOHYDRATES	
	MINUS S	PLUS S	MINUS S	PLUS S	MINUS S	PLUS S	MINUS S	PLUS S
Upper.....	5.005	19.592	0.365	2.472	1.296	0.725	0.516	0.323
Middle.....	3.660	16.767	0.900	2.480	0.735	0.537	0.972	0.512
Lower.....	2.246	10.730	0.747	1.738	0.315	0.098	1.305	1.095

TABLE 6  
CARBOHYDRATE FRACTIONS (GRAMS PER 100 PLANTS)

STEM LEVEL	REDUCING SUGARS		SUCROSE		STARCH		ACID-HYDROLYZABLE CARBOHYDRATES	
	MINUS S	PLUS S	MINUS S	PLUS S	MINUS S	PLUS S	MINUS S	PLUS S
Upper.....	1.209	17.744	0.088	2.238	0.313	0.656	0.124	0.292
Middle.....	1.226	20.334	0.301	3.008	0.246	0.651	0.325	0.620
Lower.....	1.470	22.550	0.488	3.652	0.206	0.205	0.854	2.301

The data for carbohydrates calculated on an absolute basis are given in table 6. The much larger plus-sulphur stems contain considerably more of each compound than the minus-sulphur. As in the case of the nitrogen fractions, there is a tendency for the percentage gradients to level out when the data are figured on an absolute basis. All the carbohydrate fractions show a definite gradient, although it is not in all cases in the same direction as when the data are figured on a percentage basis. The positive gradients of reducing sugars on the latter basis become negative; sucrose which showed no gradient on a percentage basis becomes negative; starch remains positive; and the negative gradient of the acid-hydrolyzable carbohydrates is intensified.

SULPHUR FRACTIONS.—The minus-sulphur stems contain at each level considerably more of the sulphur compounds soluble in 70 per cent alcohol than the plus-

sulphur; the sulphur not soluble in the alcohol and total sulphur are higher in the latter stems (table 7). The sulphur gradients are not so regular in all cases as are the nitrogen and carbohydrate gradients previously described, but the upper levels are considerably higher in the various sulphur fractions than the lower. On an absolute basis (table 8) the large plus-sulphur stems contain much more of each of the sulphur fractions than the minus-sulphur. When the data are figured on this basis there is the same tendency for the percentage gradients to level out that was noted for the carbohydrate and nitrogen compounds.

TABLE 7  
SULPHUR FRACTIONS (PERCENTAGE ON DRY-WEIGHT BASIS)

STEM LEVEL	70% ALCOHOL-SOLUBLE S		70% ALCOHOL-INSOLUBLE S		TOTAL S	
	MINUS S	PLUS S	MINUS S	PLUS S	MINUS S	PLUS S
Upper.....	0.154	0.063	0.130	0.302	0.284	0.365
Middle.....	0.160	0.100	0.085	0.272	0.245	0.372
Lower.....	0.052	0.057	0.068	0.115	0.120	0.172

TABLE 8  
SULPHUR FRACTIONS (GRAMS PER 100 PLANTS)

STEM LEVEL	70% ALCOHOL-SOLUBLE S		70% ALCOHOL-INSOLUBLE S		TOTAL S	
	MINUS S	PLUS S	MINUS S	PLUS S	MINUS S	PLUS S
Upper.....	0.037	0.057	0.031	0.273	0.068	0.331
Middle.....	0.053	0.121	0.028	0.329	0.082	0.451
Lower.....	0.034	0.119	0.044	0.241	0.079	0.361

## Discussion

### APPEARANCE OF PLANT: CHEMICAL COMPOSITION

The visible effects of sulphur deficiency on the sunflower are similar to those on the soybean (5): the yellow-green color and reduced size of the leaves and the thinner stems as compared with the plus-sulphur plants (fig. 1). Stem elongation is a prominent feature in both cases.

As NIGHTINGALE (21) points out, the symptoms of sulphur deficiency (especially as regards color) are similar to those caused by the deficiency of other elements (for example, nitrogen, potassium, phosphorus, and calcium), and may be explained on a similar basis, namely, poor nitrate assimilation, due in the first case of course to the lack of nitrates in the nutrient solution and in the other deficiencies to the

lowered reductase content of the plant (7). Nitrates are not assimilated properly and the result is only slight chlorophyll development.

The effects of sulphur deficiency on the chemical composition of the sunflower stems were also similar to those of the soybean (5). The soluble organic nitrogen compounds—ammonia, amide, and amino acids—were much higher in the minus-sulphur stems than in the plus (table 3). The soluble sulphur fractions also accumulated in the sulphur-deficient stems (table 7). These fractions were not determined



FIG. 1.—Sunflower plants: plus-sulphur and minus-sulphur

in the case of the soybean. Nitrates and the carbohydrates—starch and acid-hydrolyzable carbohydrates—also were higher in the sulphur-deficient stems, but the sugars were lower (tables 3, 5). Nitrates, starch, and acid-hydrolyzable carbohydrates no doubt accumulated in the minus-sulphur stems, due to the fact that protein synthesis for which these compounds are needed proceeded slowly because of the low reductase content of the plants (7).

The carbohydrate situation resulting from sulphur deficiency varies in different plants. All forms of carbohydrates accumulate in a minus-sulphur tomato stem (21). While a sulphur-deficient soybean stem is lower in sugars than a plus-sulphur stem, it may be enough higher in polysaccharides to be high-carbohydrate when all forms of carbohydrates are included (5). On the other hand, although the minus-sulphur sunflower stems are slightly higher in starch and acid-hydrolyzable

carbohydrates, they are so much lower in sugars than are the stems of the plants grown with complete nutrient that they are to be considered low-carbohydrate. As already stated, the accumulation of carbohydrates, and also nitrates, in sulphur-deficient plants is to be accounted for by the low protein synthesis resulting from their low reductase content. It is not known why reduced protein synthesis causes the accumulation in the tomato of all forms of carbohydrates, but in the soybean and sunflower only of the polysaccharides.

#### REUTILIZATION

It was stated at the beginning of this paper that minus-sulphur plants through proteolysis seem to secure organic nitrogen and sulphur compounds for use in stem elongation. This section discusses various lines of evidence for this theory of reutilization, and refers to another process that may account partly for the accumulation of soluble organic nitrogen and sulphur compounds in a sulphur-deficient plant.

GRADIENTS.—As regards deficiencies other than sulphur, certain work indicates transfer of materials from the older parts of the plant to the younger. For example, the investigations on barley by RICHARDS and TEMPLEMAN (26) indicate that deficiency of phosphorus, potassium, or nitrogen resulted in the transfer of the respective element from the old leaves to the meristem. MASON and PHYLLIS (15) showed that during nitrogen deficiency the younger leaves of cotton increased their nitrogen content at the expense of the older leaves at the base of the plant. Also, carbohydrates and organic nitrogen compounds seem to be translocated on a gradient basis; that is, from a region of greater concentration to one of less. This has been more especially demonstrated for downward transport of carbohydrates (16).

In the present work the sunflower stems were analyzed at three levels with the idea that the gradient data might give some indication of whether there was marked proteolysis in the lower part of the minus-sulphur stem and upward transport of the resulting compounds. If these processes are going on rapidly, it might be expected that the gradients of the nitrogen and sulphur compounds would be positive upward. But as shown by tables 3 and 7, the gradients give no support to this theory of upward transport. The gradients of all the nitrogenous and sulphur compounds are negative upward rather than positive upward, although the gradients of the latter compounds are not so regular as the former.

For a number of reasons, however, the nature of the gradients should not be overemphasized as evidence against upward transport of the compounds just mentioned. One reason is that, although organic nitrogen compounds seem to be translocated on a gradient basis, it may not be easy to prove this, as MASKELL and MASON (16) found. Although their work had shown that carbohydrates were

translocated down the cotton stem in the bark along a marked positive gradient, and although their preliminary work (13) indicated that nitrogen was also transported downward in the bark on a gradient basis, determination of the gradients (14) showed that nitrogen was moving downward against a gradient of organic crystalloid nitrogen. It was suggested that the gradient of the mobile form of nitrogen was positive downward but that this was masked in some way, perhaps by a greater gradient in the reverse direction of static storage nitrogen, so that the net gradient was negative downward. Later work (15) gave support to this masking effect of storage nitrogen. Perhaps a similar masking effect operates in the present work to cause the gradient to be negative upward rather than positive upward.

Another reason for not necessarily regarding the gradients as evidence against upward transport of nitrogenous and sulphur compounds is that my analyses are rather limited. Only one analysis was made and that rather late in the vegetative period. The stem sections were analyzed as a whole; they were not divided into different tissues. Also no ringing experiments were performed.

It is possible that earlier in the vegetative period the gradients were positive upward, but that owing to transport of nitrogenous and sulphur compounds from the base of the plant to the top they became negative, as MASON and PHYLLIS (15) found for nitrogen in cotton stems. If this were true in the sunflower, analyses at intervals would have demonstrated it. Since most of the evidence indicates that organic compounds are translocated in the phloem, the bark should have been analyzed separately from the other tissues. The gradient in the bark might have been masked by a greater reverse gradient in the other tissues. If there is upward transport of nitrogen and sulphur, then a ring near the top would cause an accumulation of these compounds below the ring. In the case of the cotton stem, MASKELL and MASON (13) found that nitrogenous compounds accumulated above a ring, indicating downward transfer. They determined the gradients in bark and wood separately (14).

After full consideration of the preceding points, however, it must be admitted that the gradient data do not support the theory of proteolysis in the lower parts of the minus-sulphur stem and transfer of the resulting material to the top to be used in stem elongation. Further work is needed, including ringing experiments, before a definite conclusion can be made.

Of course, since the gradients of the sulphur-deficient stems are positive downward, they might be interpreted as indicating synthesis in the upper parts of the minus-sulphur plants and transfer downward of the organic nitrogen and sulphur compounds. But synthesis is no doubt at a minimum in these plants because of the probably low reducase content and lack of sulphur. This interpretation would not seem to apply, therefore, although in the absence of ringing experiments no positive statement can be made. And since the gradients are in the same direction in

the plus-sulphur stems where synthesis is going on rapidly, as in the minus-sulphur stems where it is not, the gradients of the former should also probably not be interpreted as indicating downward transfer.

The gradients of nitrogenous and sulphur compounds are in the same direction as that of metabolically active cells; both are positive downward. It would seem that the positive gradients of these compounds merely result from the metabolic processes of these cells, the compounds composing the gradients being used in or resulting from these processes. This was ENGARD'S (9) interpretation of the positive gradients of nitrogenous compounds in the vegetative canes of the raspberry growing in soil. Nitrates are distributed freely in the canes; they are available in all living cells for the synthesis of organic, nitrogenous compounds. Since the number of living, protoplasm-containing cells is greater in the upper parts of the canes than in the lower, synthesis goes on more rapidly there; hence the positive gradients. That the direction of the gradients did not indicate translocation downward was indicated by the failure of a ring to reverse the gradient or even to make it level. A similar explanation would seem to apply to the sunflower. As shown by table 3, the nitrate gradient is positive downward. This is the same as the gradient of living cells, so nitrates are no doubt available for synthesis of organic nitrogenous compounds in these cells. And synthesis probably accounts mainly for the positive gradients of the plus-sulphur stems. But in the case of the minus-sulphur stems, since synthesis is probably at a minimum because of low reducase content and lack of sulphur, proteolysis rather than synthesis is probably more important in accounting for the gradients of the soluble organic nitrogenous and sulphur compounds. If there is little upward transfer of the materials resulting from the proteolysis, then the extent of proteolysis itself is represented by a gradient which is positive downward; in other words, the proteolysis may go on mainly in the place where resulting compounds are found, with perhaps transfer through only short distances. LEONARD (10), in a study of field-grown sunflower plants, also found positive downward gradients of nitrogenous compounds in the stems. His data indicate that synthesis of these compounds took place mainly in the young leaves, the upper pith, and the head.

This section is not primarily concerned with the carbohydrates. It is interesting, however, that there is the same correlation between reducing sugars and starch (table 5) and protoplasm-containing cells already noted for the nitrogenous and sulphur compounds; the gradients of these carbohydrates and of the living cells are both positive downward. And perhaps a similar explanation may be given for this correlation as was given in the preceding paragraph in the case of the nitrogen and sulphur compounds. ENGARD (8) found that all the carbohydrate gradients (with the possible exception of sucrose) in the raspberry canes were positive downward and explained these mainly on the basis of temporary storage in living cells,

or in the case of the polysaccharides, cells associated with living cells, although part of the gradient of reducing sugars was translocatory, as shown by ringing experiments. This explanation probably holds for the reducing sugars and starch of the present experiment. As shown by table 5, while the starch gradient is positive downward the acid-hydrolyzable gradient is negative downward. Sucrose exhibits no gradients.

ACCUMULATION OF AMIDE NITROGEN.—The gradients are hard to interpret and cannot be used as definite evidence of proteolysis and reutilization. But that proteolysis is going on, especially in the minus-sulphur stems, is indicated by the accumulation in these stems of the soluble organic-nitrogen fractions of which amides form an abnormal percentage. As shown by table 3, the fractions ammonia, amino acids, and amides are several times higher in the minus-sulphur stems than the plus-sulphur, and amides make up a much greater proportion of the soluble nitrogen of the former stems than of the latter. Only a trace of amide was found in the plus-sulphur stems. Table 7 shows that soluble sulphur is also considerably higher in the minus-sulphur stems. The soluble sulphur fraction includes not only organic sulphur but also any sulphate sulphur that may be present. It would have been interesting to have fractionated the soluble sulphur into sulphate sulphur and organic sulphur. NIGHTINGALE (21) got indications of the proteolytic formation of sulphates in germinating seeds, and WOOD and BARRIEN (37) report that under conditions of starvation in the dark protein sulphur decreases and is accompanied by an equal increase in sulphate sulphur.

The preceding situation, at least as regards the nitrogen fractions, usually prevails when proteolysis is taking place in plants. The germination of seeds affords a familiar illustration. During germination the carbohydrates decrease greatly, the proteins are hydrolyzed to amino acids and amides, part of the amino acids is oxidized (ammonia being produced), and the ammonia is combined with carbon compounds arising from this oxidation of the amino acids (or from carbohydrates) to give amides. While part of the amide pre-existed in the seed proteins, most of it is of secondary origin, as just described; there is much more amide in the seedling than was found in the proteins of the seed. This secondary production of amide is at present regarded as part of the respiratory process. Usually it does not go on rapidly in the plant until the carbohydrates have become too deficient to supply the necessary energy for the plant's life processes. In this situation energy is obtained by the oxidation of amino acids. CHIBNALL (2), VICKERY (34), NIGHTINGALE (22), and MURNEEK (18) discuss proteolysis and amide metabolism.

Proteolysis in plants is a common phenomenon. Besides the germination of seeds, it has been studied especially in detached leaves (2, 33, 34) and in plants in the dark (19). In discussions of proteolysis, however, mineral deficiencies usually are not included. Yet on theoretical grounds it might perhaps be expected that

when an element is deficient, proteolysis would occur. As RICHARDS and TEMPLEMAN (26) point out, it does not seem likely that a deficient element would be found in the plant in a form not utilizable in metabolism; that is, it might be expected that when the supply of an element is deficient organic compounds of this element would be broken down, releasing the element in a form that could be used in metabolism. And, as previously mentioned, the work of these investigators on nitrogen, phosphorus, and potassium deficiencies indicated transfer of these elements from the old leaves of the barley to the meristem. Also, analyses of the nitrogen-deficient cotton plant showed transfer of nitrogen from the old leaves to the young (15).

Although doubtless mineral deficiency commonly causes proteolysis, the process does not seem rapid, at least in the case of the deficiencies being considered, unless the plant is put in the dark or there is injury to the protoplasm, as in advanced stages of phosphorus or potassium deficiency (21). In certain other deficiencies (for example, calcium and boron) proteolysis and reutilization may be even slower. NIGHTINGALE (20) found that the root and stem apices of calcium-deficient tomato plants died while there was still much calcium in the forms of calcium oxalate crystals and combined calcium in the older tissues. Boron deficiency also affects the meristematic tissues of the apices of the plant. The fact that these deficiencies, including nitrogen, do not normally result in rapid proteolysis may be at least partly conditioned by the fact that they result in the accumulation of carbohydrates. High carbohydrate content favors synthesis rather than hydrolysis of proteins.

At first sight at least, proteolysis in sulphur-deficient plants seems to constitute a rather special case. It is usually correlated with a reduction of carbohydrates. Sulphur deficiency does not seem to injure the protoplasm (21). Yet hydrolysis of the proteins goes on rapidly in a minus-sulphur plant while it is in the light and even though it is high in carbohydrates. Thus there are provided organic sulphur and nitrogen compounds which would otherwise be deficient because of the lack of sulphur in the nutrient solution and the effect of sulphur deficiency in causing a low reducase content of the plant. And the sulphur-deficient plant elongates rapidly in contrast with the other deficiencies just mentioned.

Although proteolysis in a sulphur-deficient plant seems to be abnormal, in that the process goes on rapidly even though the plant is high in carbohydrates, yet sulphur-deficient plants vary greatly as to the carbohydrate situation. All forms of carbohydrates accumulate in a minus-sulphur tomato plant (21). The soybean is decidedly lower in sugars but may be enough higher in polysaccharides to be considered high-carbohydrate in comparison with the plus-sulphur plant (5). While the sunflower is slightly higher in polysaccharides, it is so low in sugars that it should be regarded as low-carbohydrate (table 5). So proteolysis in the soybean



and the sunflower, and especially in the latter, may be considered more in line with that usually going on in plants than is true of the tomato. Proteolysis in the soybean and sunflower is associated with a reduction in the more available carbohydrates, reducing sugars and sucrose; in the tomato all forms of carbohydrates accumulate. But considering all three plants together, it would seem that the concentration of carbohydrates is not the factor controlling proteolysis.

A similar statement may be made in regard to other more commonly studied cases of proteolysis; for example, detached leaves. It is not known definitely what regulatory mechanism controls synthesis and hydrolysis of proteins. Usually high carbohydrate content favors synthesis, low carbohydrate content, hydrolysis; the "sparing action" of carbohydrates is a well-known phenomenon. But this is not always the case. Rapid proteolysis may go on under conditions which maintain a rather high level of carbohydrate or even cause a decided increase in the concentration; as for example, in the case of some of the experiments with detached leaves in the light or in glucose solutions in the dark (2, 33, 34). So the carbohydrate content does not give an adequate explanation. As CHIBNALL (2) points out, "We do not yet understand the reason why, when certain leaves are detached from the plant, protein decomposition can be detected within a few hours." CHIBNALL gives an excellent discussion of the various theories regarding regulation of protein metabolism in leaves. A similar statement may perhaps be made in regard to sulphur-deficient plants; it is not known why, when sulphur is deficient, proteolysis occurs. But some factor other than the carbohydrate concentration would seem to be controlling the process.

It would seem, therefore, that proteolysis in sulphur-deficient plants should not after all be considered so different from other cases of proteolysis. Proteolysis may go on in sulphur-deficient plants of widely varying carbohydrate concentration; the same is true of other more generally discussed types of proteolysis.

**NEED OF SULPHUR FOR PROTEIN SYNTHESIS.**—Calculations reported in previous work on the soybean (5) indicate that the need of sulphur for the synthesis of proteins from amino acids and amides accounts partly for the accumulation of soluble organic nitrogen compounds in a sulphur-deficient plant. The calculations involved comparing the organic nitrogen content of seed and plant. Similar calculations in the case of the sunflower lead to the same conclusion. In the present work the seed was not analyzed, but seed from the same lot has been used in class experiments in the laboratory. The seed contains 4.5 per cent total nitrogen.<sup>1</sup> The average weight of a seed is 62 mg. Assuming no nitrate present, each seed contains 2.79 mg. of total organic nitrogen. Table 4 records the data of the nitrogen fractions on an absolute basis for each level. If the organic nitrogen for the entire stem is determined, it will be found that each stem, not counting leaves and roots,

<sup>1</sup> Miss DAPHNE SWARTZ kindly supplied the determinations of total nitrogen and weight of seed.

contains more than eight times as much total organic nitrogen as the seed from which the plant developed. There must have been synthesis of much organic nitrogenous compounds in the sulphur-deficient stems. There was, of course, synthesis of much more of these compounds in the plus-sulphur plant, as shown by the table.

Synthesis of amino acids is reduced in a sulphur-deficient plant because of low reductase content, as has already been mentioned. Sulphur deficiency does not, however, interfere with reduction of nitrates as much as other deficiencies (7), so no doubt there is a constant, slow synthesis of amino acids. But sulphur-containing amino acids cannot be synthesized because of the lack of sulphur in the nutrient solution. Since nearly all plant proteins contain sulphur, the nonsulphur-containing amino acids presumably cannot be condensed to proteins; hence they accumulate in a minus-sulphur plant. In line with this viewpoint is NIGHTINGALE'S (21) observation that very little of the soluble organic sulphur of the sulphur-deficient tomato plants was cysteine or glutathione. It would seem, therefore, that part of the accumulation of soluble organic nitrogenous compounds in a minus-sulphur plant is due to the need of sulphur for the synthesis of proteins from amino acids and amides.

SUMMARY OF REUTILIZATION.—Proteolysis seems to proceed readily in a minus-sulphur plant, even though it is high in carbohydrates. This is indicated mainly by the accumulation of soluble organic sulphur and nitrogenous compounds, an abnormal percentage of the nitrogenous compounds being amides. Thus there are provided these compounds under conditions which would otherwise cause them to be quite deficient, that is, low nitrate assimilation and the lack of sulphur in the nutrient solution. As a result, stem elongation occurs rapidly. Although the gradient data are limited, the indications are that the proteolysis proceeds mainly in or near the place where the soluble compounds are found, with perhaps transfer through only short distances. There may be no transport of materials from the base to the tip of the plant. Part of the accumulation of the soluble nitrogenous compounds is probably due to the need of sulphur-containing amino acids for the condensation of amino acids to proteins.

#### IMPORTANCE OF ACID-HYDROLYZABLE CARBOHYDRATES IN METABOLISM

This work has not been concerned primarily with a study of the importance of the acid-hydrolyzable carbohydrates in the metabolism of the sunflower. However, a consideration of the carbohydrate data (table 5) shows that, while the starch and reducing sugar gradients are in the same direction as the metabolically active cells, the gradient of the acid-hydrolyzable substances is in the reverse direction and corresponds with the gradient of nonliving cells. Since starch is always regarded as readily usable by the plant, the fact that the acid-hydrolyzable gradi-

ent is in the reverse direction might be regarded as indicating that under the conditions of this experiment the acid-hydrolyzable carbohydrates of the sunflower are not usable, or at least not so much so as starch. Since only one analysis was made, however, no definite conclusions can be drawn.

It is a moot question whether the acid-hydrolyzable carbohydrates are used in metabolism or whether they should be considered as rather permanent structural constituents of the cell wall. CLEMENTS (3) is inclined to regard these compounds as readily usable by the plant. On the other hand, WINKLER and WILLIAMS (36), after a critical review of the literature, conclude that—except in the case of the seeds of a few plants—there is no definite evidence that these substances function as reserve materials. NIGHTINGALE (19) takes the somewhat intermediate viewpoint that these carbohydrates are probably not used by the plant until starch, dextrin, and sugars have become almost depleted.

The acid-hydrolyzable carbohydrates constitute a very heterogeneous group of carbohydrates, whose importance in metabolism varies, depending on a number of factors. The nature of these carbohydrates differs in different plants and in the same plant in different tissues and under diverse climatic conditions. Also their usability is probably affected by other more readily available carbohydrates. CLEMENTS (3) concluded that under favorable conditions of growth the acid-hydrolyzable carbohydrates of the leaves of the sunflower, potato, and soybean were serving as important food reserves, since they fluctuated widely during the day; lack of fluctuation under drought conditions indicated that they were not being used in metabolism. LEONARD (10) found much greater variation in the acid-hydrolyzable material of the bark of field-grown sunflowers during the season than of the wood, indicating greater usability in the former case. ENGARD (8) states that the function of acid-hydrolyzable substances "in metabolism is variable in plants and depends to a great extent upon the kinds and amounts of other more readily utilizable carbohydrates." He found very little starch in the raspberry canes, and his work indicated that the acid-hydrolyzable substances were more important than starch in the metabolism of the raspberry. These substances were removed in two fractions, one by 3 hours' hydrolysis, the other by 12 hours' further hydrolysis. The first was regarded as more important in metabolism. The sunflower plants of this experiment also contained little starch (table 5), yet there are indications that the starch is more related to metabolism than are the acid-hydrolyzable substances. Analyses should have been made at intervals, however, to compare the fluctuations in the amounts of starch and the acid-hydrolyzable material.

It would seem impossible to make any categorical statement as to the importance of the acid-hydrolyzable carbohydrates in metabolism. Sometimes they are important, sometimes they are not. Their usability varies with the kind of

plant, carbohydrate, tissue, and climatic conditions, with the amounts of other carbohydrates, and perhaps other factors. Data indicating lack of importance in metabolism should not be overemphasized. As NIGHTINGALE (19) points out, it is hardly fair to use data obtained by acid hydrolysis as a criterion of what the enzymes of the plant can do. And finally, as CLEMENT'S (3) work indicates, even if the acid-hydrolyzable carbohydrates are not important in metabolism, they may have other important functions, such as tissue strengthening and protection from drought.

### Summary

1. The visible effects of sulphur deficiency in the sunflower were similar to those noted in previous work on the soybean: the yellow-green color of the leaves, the smaller leaves, and the thinner stems as compared with the plus-sulphur plants. Stem elongation was a prominent feature.

2. The soluble organic nitrogen fractions and nitrates accumulated in the minus-sulphur stems as compared with the plus-sulphur. This was also true of the soybean. Soluble sulphur compounds were also higher in the sulphur-deficient sunflower stems. These were not determined in the case of the soybean.

3. While the minus-sulphur sunflower stems were somewhat higher in starch and acid-hydrolyzable carbohydrates than the plus-sulphur, they were so much lower in sugars that they were to be considered low-carbohydrate stems. On the other hand, although the sulphur-deficient soybean stems were lower in sugar than the plus-sulphur, they were so much higher in the polysaccharides that they were to be regarded as high carbohydrate. All forms of carbohydrates accumulate in a minus-sulphur tomato stem. So plants vary in this regard.

4. The accumulation of nitrates and carbohydrates in minus-sulphur plants seems to be due to low nitrate assimilation, which results from the low reductase content of the plants. The low nitrate assimilation also no doubt accounts for the yellow color of the sulphur-deficient leaves, as is true of other deficiencies; for example, potassium, calcium, and phosphorus. Accumulation of soluble organic nitrogen fractions and soluble sulphur compounds seems to be due mainly to proteolysis. Proteolysis and reutilization seem to be more prominent features of sulphur deficiency than is true of most other deficiencies.

5. Part of the accumulation of the soluble organic nitrogen fraction as a result of sulphur deficiency may be due to the lack of sulphur-containing amino acids for the completion of protein synthesis.

6. It is not known why low protein synthesis results in the accumulation in the tomato of all forms of carbohydrates but in the soybean and sunflower of only starch and acid-hydrolyzable carbohydrates.

7. Since the gradients of the nitrogen and sulphur fractions are negative upward rather than positive upward, they offer no support for the theory of proteolysis

in the lower levels of the sulphur-deficient stems and transport of the resulting materials to the tips to be used in stem elongation.

8. Since only one analysis was made (and that rather late in the vegetative period), the different tissues were not analyzed separately, and no ringing experiments were performed, perhaps the nature of the gradients should not necessarily be regarded as evidence against upward transport.

9. It may be, however, that proteolysis in the minus-sulphur stems goes on at or near the place where the resulting materials are found, with transfer at the most through only short distances.

10. The gradients of nitrogenous and sulphur compounds were in the same direction as that of the metabolically active cells, namely, positive downward. It seems likely that the gradients result from the metabolic activities of these cells: proteolysis in the case of the sulphur-deficient stems, synthesis in the case of the plus-sulphur.

11. The positive downward gradients of starch and reducing sugars may also result from synthesis in living cells, although in this case the gradients may be partly translocatory.

12. The gradients in themselves do not afford evidence for the theory of proteolysis and reutilization. But the accumulation in the minus-sulphur stems of soluble organic nitrogen fractions, of which amide forms an abnormal percentage, indicates that rapid proteolysis is going on in these stems. This situation usually prevails in proteolysis.

13. Proteolysis in a sulphur-deficient plant seems abnormal in that it proceeds even though the plant is high in carbohydrates. Usually proteolysis is associated with a reduction in carbohydrates. But it would seem that carbohydrate content is not the controlling factor in proteolysis. The process may go on in detached leaves, for example, under conditions of either high or low carbohydrate content. The same may be said for minus-sulphur plants. The sulphur-deficient tomato and soybean stems may be regarded as high carbohydrate, although sulphur deficiency results in a lower sugar content of the soybean stems. On the other hand, a minus-sulphur sunflower stem is to be considered low in carbohydrates although slightly higher than the plus-sulphur in starch and acid-hydrolyzable carbohydrates. From this standpoint proteolysis resulting from sulphur deficiency should not be considered so abnormal.

14. While the starch and reducing sugar gradients of the sunflower stem are in the direction of the metabolically active cells, positive downward, the acid-hydrolyzable gradient is in the reverse direction, that of the nonliving cells. This may be regarded as evidence that under the conditions of this experiment the acid-hydrolyzable carbohydrates are not so important in metabolism as starch and reducing sugars.

15. No categorical statement can be made as to the importance of the acid-hydrolyzable carbohydrates in metabolism of plants. Their usability may vary with the kind of plant, carbohydrate, climatic conditions during growth, with the amount of other more available carbohydrates present, and perhaps other factors.

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# EFFECTS OF SOME ENVIRONMENTAL FACTORS ON PHOTOPERIODIC INDUCTION OF BEET AND DILL<sup>1</sup>

CONTRIBUTIONS FROM THE HULL BOTANICAL LABORATORY 521

AUBREY W. NAYLOR

(WITH SIX FIGURES)

## Introduction

Among other factors of the environment, temperature and photoperiod are important in influencing the disposition of many plants to differentiate floral primordia. This is particularly true for plants of a biennial habit. A number of investigators (2, 8, 14, 18, 19, 20) agree that the promotive effects of low temperature in initiating seed-stalk development may be subsequently decreased at higher temperatures. Inasmuch as the temperature phases of many photoperiodically sensitive plants may be very short and yet effective in influencing subsequent response to light, as is well illustrated by vernalization experiments, care must be exercised in interpreting results in terms either of light reaction or of reaction to temperature.

If an interpretation is to be made in terms of photoperiodic reaction alone, it is necessary to select plants which do not require a temperature phase in order to render them sensitive to the proper photoperiod for flowering. Since some of the work reported here was performed to determine whether the theories of floral initiation which have been advanced (4, 12, 5) can be used to interpret the flowering response of long-day plants, the plants used were not temperature limited.

Dill (*Anethum graveolens* Linn.) was previously reported (7) to be a sensitive long-day plant having a short induction period and the ability to produce quickly a flowering stalk after four long photoperiods when returned to short photoperiod conditions. MURNEEK (13) has recently emphasized the sensitivity of dill, and in addition has pointed out that it, like Biloxi soybean, will flower if kept for sufficient time on photoperiods not considered conducive to flowering. Previous work had also shown that dill did not require a low temperature treatment in order to be photoperiodically sensitive.

The variety of beet (*Beta vulgaris* L.) chosen differs from most varieties in that it does not have a definite temperature phase. There has been very little modifica-

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tion in its flowering response over rather wide temperature ranges in the greenhouse. Another characteristic of this variety is its capacity to flower vigorously after fourteen or more long photoperiods (19–21 hours of light and 5–3 hours of darkness) when returned to short photoperiod (8 hours of light out of 24).

The dill seeds were obtained from commercial sources; the beet seeds were obtained from Dr. E. Carsner, Riverside, California.

### General methods

No attempt was made to regulate either the temperature or the humidity of the greenhouse, other than to prevent excessively high or low temperatures. The rooms were heated whenever the temperature fell below 18° C. and ventilated when temperatures rose above 32° C. Humidity was partially controlled by spraying the walks and walls with tap water. The plants were watered twice daily with tap water and at intervals with a modified SHIVE's (16)  $R_2S_5$  nutrient solution.

The seeds were germinated in flats containing a light garden soil. After planting the seeds, the flats were immediately placed either on benches or trucks and subjected to cycles of the desired photoperiod. The beets were usually germinated on shallow greenhouse benches of the usual type, except that they had low rectangular wooden frameworks arranged over them. These frames supported a double thickness of black sateen cloth which was placed over them nightly to control the length of the photoperiod. The dill seeds were germinated on small trucks which were rolled into light-tight sheds each evening.

In the course of the work with dill, it was found necessary to transplant the very young seedlings from flats to  $3\frac{1}{2}$ -inch pots. If the leaves had attained a length of 4–5 inches and there were more than three or four expanded leaves present at the time of transplanting they were considered unreliable test plants, since they tended to flower spontaneously even though maintained on cycles of short photoperiod. The cause of this tendency to flower has not been investigated critically, but there is some evidence that injury to leaves and roots during the transplanting process may in some way alter the metabolism of the plant sufficiently to bring about flowering. Further evidence to support this viewpoint is given later. All dill plants were maintained on a 9-hour photoperiod until they were used experimentally.

The beet seedlings were transplanted singly to  $3\frac{1}{2}$ -inch pots when their leaves were 4–6 inches long. After transplanting they were returned to the short photoperiod benches. These benches were covered each evening at 4:00 P.M. with the black sateen cloths and were uncovered the following morning at 8:00 A.M., thus affording 16 hours of darkness and 8 hours of light.

The response considered of primary importance in both plants was the change from the rosette condition of the main axis to elongation of the stem or seed stalk.

Elongation of the stem was not always accompanied by production of normal, fertile flowers. In some instances beet stems would be slightly elongated but fail to produce flowers, or if flowers were formed they were reduced in size and sterile. This type of response was usually the result of what was considered incomplete induction. In all the experiments with dill, rapidity of response was measured in terms of the average length of the stems at the time of harvest. Dill plants were usually harvested in any given experiment when the inflorescences of the plants receiving any one treatment began to unfold.

### Investigation

#### EXPERIMENTS WITH DILL

The experiments were planned to test the effects on vegetative growth and reproductive response of (a) continuous illumination of varying intensities from time of germination; (b) continuous illumination of relatively constant intensity from time of germination; (c) interrupting the dark portion of the cycle with light for different lengths of time; (d) high light intensity while on short photoperiod, followed by high intensity on long photoperiod or by low intensity on continuous photoperiod; (e) low light intensity followed by intensities varying from 50 to 1000 foot-candles; (f) different intensities of red light on number of days necessary for induction; (g) low temperature during induction period on flowering response. Another experiment was performed in order to determine the effect of certain types of injury on subsequent development of dill.

CONTINUOUS ILLUMINATION OF VARYING INTENSITIES FROM TIME OF GERMINATION.—To determine whether dill will germinate and grow to maturity under conditions of continuous illumination of high intensity, seeds were germinated in the greenhouse April 18, 1940. These seeds were scattered on the surface of the soil, which was kept moist by frequently sprinkling with a fine spray. Natural daylight was supplied from 8:00 A.M. to 5:00 P.M., at which time a reflector containing six 36" Mazda fluorescent lamps of the daylight type was lowered into position over the seeds. The lamps, affording approximately 750 foot-candles at the soil surface, were used until the following morning at 8:00 A.M., when they were removed.

Germination began approximately 1 week after planting. Instead of the typical rosette type of growth, the stems elongated but grew only slightly in diameter. Approximately 40 days later the plants had attained a height of about 10 cm. and began unfolding their inflorescences. Since the light intensity during the first half of the experiment was more than 700 foot-candles and never fell below 200 foot-candles, it is evident that neither darkness nor light of very low intensity is necessary for the flowering response of dill.

CONSTANT ILLUMINATION AT RELATIVELY CONSTANT INTENSITIES FROM TIME OF GERMINATION.—This experiment was set up in a darkroom where the sole source

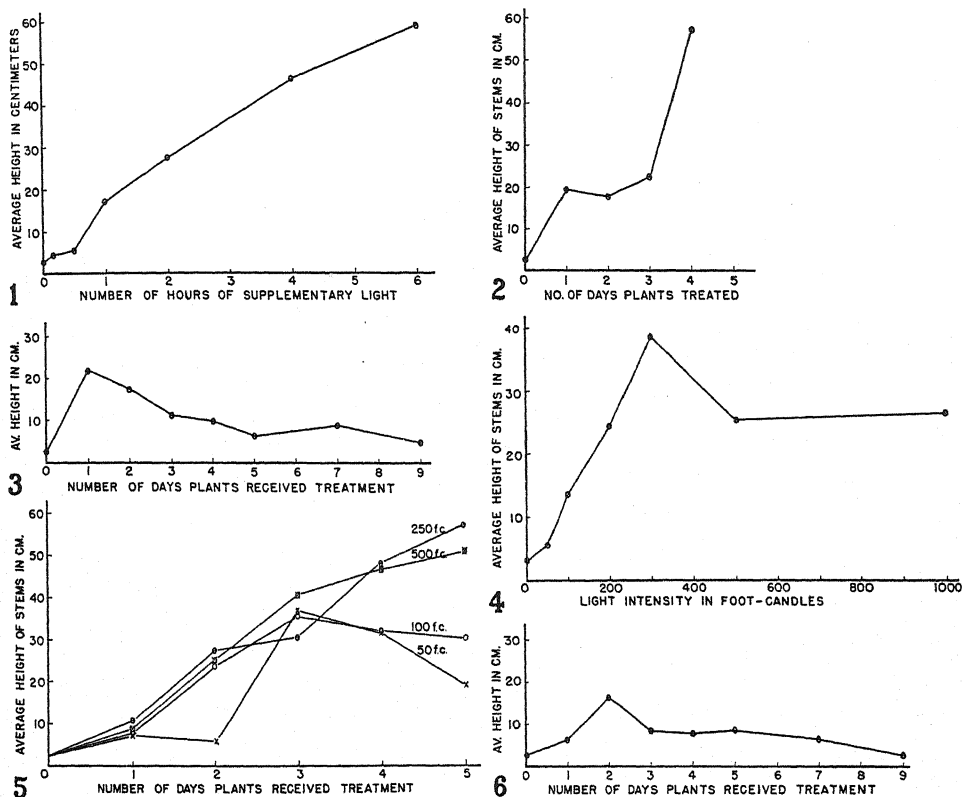
of illumination was from Mazda lamps of the daylight fluorescent type. One lot of seeds was planted at the surface of the soil, where light intensity was adjusted to approximately 1000 foot-candles. Another lot was planted similarly except that the light intensity was adjusted to approximately 500 foot-candles. The lamps burned constantly during the experiment. There was, however, a gradual increase in intensity at the surface of some of the leaves as the plants grew, since the reflectors were never readjusted. The highest intensity registered under the first reflector was approximately 1400 foot-candles and under the second, 750 foot-candles.

The plants germinated under the highest intensity grew rapidly and were sturdy. For about 30 days they remained as rosettes. After 40 days, stems of a few were elongating. Thereafter many others began to respond. Inflorescences appeared when the plants were 15-20 cm. tall. In contrast, the plants germinated under the lower light intensity grew slowly, were more succulent, and were easily bruised. They remained in the rosette stage for approximately 2 months. Several plants then began to elongate and continued to until the conclusion of the experiment September 10, when sixty-eight plants had elongated to some extent. Some stems attained a length of 50 cm. before inflorescences appeared but were bent over because of weakness.

INTERRUPTING DARK PHASE OF CYCLE WITH LIGHT FOR DIFFERENT LENGTHS OF TIME.—It has been reported that dill requires at least 14 hours of light per day for about 4 days to result in stem elongation. Inasmuch as some short-day plants react as though on long photoperiod when they are given short cycles of light during the dark period (5, 17, 10), tests were made to determine the response when the dark period was interrupted by an interval of light. Three hundred uniform dill plants  $2\frac{1}{2}$  months old were distributed approximately equally in seven small framework compartments. All the plants received daylight 9 hours out of each 24. The plants were then darkened and supplied in six of the compartments with Mazda filament light of approximately 100 foot-candles at the leaf surface, the amount of light being automatically controlled by time clocks. Six different photoperiods were given in the middle of the dark period, ranging from 10 minutes to 6 hours. The plants in one compartment remained in darkness and served as controls. Treatment was continued for 40 days, at which time the plants receiving 6 hours of light as an interruption of the dark period were apparently at their maximum height and were beginning to shed pollen. Measurements of the length of stems of all the experimental plants were then made and averages taken (fig. 1).

Groups of plants having the dark period interrupted for either 10 minutes or 30 minutes elongated no more than the controls. In contrast, those plants receiving 1 hour of supplemental illumination (that is, a total of 10 hours of light out of each 24) elongated several centimeters during the treatment. This point is of particular

interest since the total amount of light during these 10 hours was 4 hours less than is required for stem elongation if the photoperiod is given uninterruptedly. With increasing duration of illumination (fig. 1) during the dark period up to 6 hours, the average height of the plants was almost directly proportional to the amount of supplementary illumination received.



FIGS. 1-6.—Dill: Fig. 1, effect on stem elongation of supplementary light of approximately 80 foot-candles given during middle of dark period (9 hours of daylight each day; measurements taken 40 days later). Fig. 2, response of plants receiving indicated number of cycles, each consisting of 20 hours of light and 4 of darkness (measurements taken 45 days later). Fig. 3, response of plants receiving continuous illumination of 4 foot-candles (measurements taken 45 days later). Fig. 4, effect of continuous light, given after plants had been subjected to 3 short days of low light (measurements taken 51 days later). Fig. 5, effect of red light of various intensities (measurements taken 60 days later). Fig. 6, response of plants receiving cycles, each consisting of 20 hours of light and 4 hours of dark at temperature 4.5° C. (measurements taken 36 days later).

HIGH INTENSITY PREVIOUS TO INDUCTION FOLLOWED BY HIGH INTENSITY DURING INDUCTION.—Under light conditions prevailing in January and February at Chicago, dill has an induction period of 4 days (7). Since light intensity plays an im-

portant role in floral initiation (1, 5), an experiment was performed to determine whether the average higher light intensity of June would appreciably alter the number of days required for induction. On June 15, 1939, fifty-two plants were placed on long photoperiod, while thirteen plants remained on short photoperiod as controls. The long photoperiod, consisting of 20-21 hours of light and 4-3 hours of darkness, was obtained by supplementing the ordinary day length with 80-100 foot-candles from Mazda filament lamps until 2:00 A.M. On each day for 4 days, lots of thirteen plants were transferred from the long photoperiod to conditions of short photoperiod, and the stems measured 44 days after the start of the experiment. Average heights of each group are given in figure 2.

The average height of those plants receiving one, two, and three long photo-inductive cycles was about 20 cm., whereas the average height of those receiving four was approximately 60 cm., about three times the average height of the plants having fewer long photoperiods.

Since plants of which the axes begin to elongate almost invariably flower, it is evident that dill can be induced to flower during periods of high light intensity, if given only one long photoperiod. Speed of elongation and production of inflorescences are much less rapid, and total height attained at the time of flowering is usually less, than in plants receiving four long photoperiods.

**HIGH LIGHT INTENSITY PREVIOUS TO INDUCTION FOLLOWED BY LOW INTENSITY DURING INDUCTION.**—To test further the effect of light intensity and the duration of light in relation to the rosette and non-rosette types of growth, the following experiment was performed. On June 15, 1939, 104 plants which had been grown on short photoperiod were selected and divided into eight groups of thirteen each. Of these, the plants composing seven groups were transferred at 5:00 P.M. to a room where the light intensity (derived from a Mazda filament source) was 4 foot-candles at the surface of the leaves and was maintained at a practically constant temperature of 26° C. The thirteen plants of the remaining group were maintained on short photoperiod as a control. One group of plants receiving continuous illumination was returned to the short photoperiod trucks each day at 5:00 P.M. for the first 5 days, another group was returned on the seventh day, and the final group was returned on the ninth day. Thirty-eight days after the last transfer was made the plants were harvested and measurements of the flower stalks made (fig. 3).

Plants receiving only 1 day of continuous illumination of low intensity grew most rapidly after being replaced on short photoperiod and attained the greatest height; those treated 2 days grew a little less rapidly; and those treated 3 days grew still more slowly. The groups receiving a greater number of days of treatment differed among themselves so little in average heights that no significance can be attached to treatment longer than four 24-hour periods.

Since light intensity previous to treatment was high, one explanation for the growth effects following the first 24 hours of continuous illumination is that the threshold value for partial induction was reached with the addition of this light, even though it was of low intensity. These plants, of course, received a high light intensity on a short photoperiod 1 day after they had had enough light for induction. If the plants received 72 or more hours of continuous light the effect was apparently negligible. Evidently the light intensity was too low for promotion of the stimulus to flower after the first 24-hour period, and the sooner the plants were returned to short photoperiod where the light intensity was higher the more they responded by elongating. Also, continued exposure to the low light intensity may have depleted the carbohydrate reserve to the point where it was impossible for the plants to recover sufficiently to produce a vigorous flower stalk. If carbohydrate reserve was a limiting factor, it was just as limiting (expressed in terms of subsequent stem elongation) after 4 days of treatment as after 9. In addition it should be noted that, shortly after returning the plants to high intensity in the greenhouse, they recovered their green color rapidly and in most cases appeared normal.

SHORT PHOTOPERIODS OF LOW LIGHT INTENSITY FOLLOWED BY INTENSITIES VARYING FROM 50 TO 1000 FOOT-CANDLES.—Since dill showed practically no further response to continuous light of low intensity after being subjected to three or more 24-hour periods, it was thought possible to remove the effects of previous treatment with high light intensity by subjecting the plants to three short photoperiods of low light intensity (4–12 foot-candles). Accordingly on September 11, 1939, an experiment was started with plants thus treated, to determine the effect of various light intensities, supplied continuously for 4 days, on subsequent development.

The 175 plants were divided into seven equal groups, 25 in each group, and subjected to different intensities of light from Mazda filament lamps, which were placed at one end of a bench in the darkroom. Six groups of plants were arranged at intensities ranging from 50 to 1000 foot-candles. The seventh group was transferred to short photoperiod at the same time for use as a control. All the plants receiving continuous light at the different intensities for four 24-hour periods were returned to short photoperiod. This was done at 5:00 P.M. in order that the plants would receive a dark period immediately following the treatments. Some plants attained their maximum height and unfolded their inflorescences 51 days after the termination of treatment. Measurements were made at that time (fig. 4).

Up to 300 foot-candles, the average height attained was almost directly proportional to the intensity of light received; the average height of the plants receiving 500 and 1000 foot-candles was almost the same as that of the plants receiving 300. There is an apparent inhibitory effect or lack of stimulation of intensities higher than 300 foot-candles on the rate of elongation of the stems.

RED LIGHT AND NUMBER OF DAYS NECESSARY FOR INDUCTION.—Some work (21) has been done on the effect of red light on photoperiodic response when used to supplement natural day length. SCHAPPELLE (15), who worked with narrow ranges of wave lengths of radiant energy in the red portion of the spectrum, demonstrated with a number of species that when red light only is used during the photoinductive cycle, floral initiation will result. In order to determine whether red light of a number of different intensities would prove equally effective in inducing stem elongation in dill, an experiment was set up June 15, 1940.

The primary source of light was provided by Mazda filament lamps screened by using four thicknesses of red cellophane stretched over sheets of glass. These filters had a transmission range below  $580\ \mu$  of 0 per cent and at  $700\ \mu$  of 64 per cent. To eliminate most of the infra-red and minimize the amount of heat reaching the plants, 2-foot square, glass-bottomed trays about 4 inches deep were placed just below the reflectors and above the red filters. Two of these trays were filled with tap water, which flowed through them constantly. Two others, used for low intensities, had their water level maintained by frequent renewals. Four different intensities were used: 500, 250, 100, and 50 foot-candles. Under each of these, groups of twenty plants each were maintained on long photoperiods, each constituting a cycle of 20 hours of light and 4 hours of darkness.

Different groups of plants received one, two, three, four, and five long photoperiods at the various intensities. Treatment began at 5:00 P.M. after the plants had received 9 hours of daylight. At the conclusion of each of the treatments the plants received 20 hours of darkness and were then returned to short photoperiod. Stem development proceeded slowly until measurements were made on September 2 (fig. 5).

The results indicate that the several intensities provided during a long photoperiod are almost equally effective in influencing stem elongation. Intensities of 100, 250, and 500 foot-candles are relatively equally effective when given in two cycles, whereas two long photoperiods consisting of 50 foot-candles of red light given for 20 hours out of each 24 were more effective than a single cycle. All four intensities were about equally effective in initiating stem elongation when the induction treatment lasted 3 days. When the plants were subjected to four long photoperiods, the plants receiving 250 and 500 foot-candles of red light averaged 43-46 cm. high, while those receiving 50 and 100 foot-candles averaged 30-33 cm. high. There appeared to be a rather steady increase in average height of plants receiving 250 and 500 foot-candles of light with increasing number of photoinductive cycles. On the other hand, those plants receiving 50 and 100 foot-candles increased less in average height after they received three photoinductive cycles, the lack of increase being greatest where the intensity was lowest.

LOW TEMPERATURE DURING INDUCTION PERIOD AND FLOWERING RESPONSE.—The temperature at which *Xanthium* plants are maintained during the dark period is an important factor in determining the number of days required for induction to flower (9). Temperature during the photoperiod apparently alters the time required for induction very little. Since dill does not require a dark period for induction, a test was made to determine whether low temperature has any influence on the number of days required for induction.

One hundred and four plants growing on short photoperiod were selected June 15, 1939. These were divided into eight groups of thirteen each. One of these groups was maintained on short photoperiod as a control. The remaining groups were transferred to a room adjusted to 4.5° C. and controlled to within 0.5° C. Light of approximately 1800 foot-candles at the leaf surface was supplied by an Everready carbon arc lamp to give a photoperiod of 20 hours out of 24. After each photoperiod for the first 5 days, and then following the seventh and ninth photoperiods, one group was returned to short photoperiod in the greenhouse. They were maintained there until July 29, when measurements of the heights of stems were made (fig. 6).

The average height of the plants having had 2 days of cold treatment was greatest of any of the groups. Plants having had 3, 4, 5, and 7 days of the cold treatment were only approximately as tall as those receiving one, whereas those receiving nine cycles reacted as did the controls remaining on short photoperiod.

RESULTS FROM INJURY TO STEM.—Initial attempts to show that there was a transmittable flower-promoting substance in dill appeared promising. Further experiments were conducted with the introduction of a greater variety of controls. The data are briefly summarized as follows. One hundred and sixty-five plants were treated March 31 and April 1 in such a way that injury to the stem always resulted. Previous to the experiment, and for its duration, plants were maintained on a 9-hour photoperiod. Fifty-one plants were in flower at the conclusion of the experiment (May 25), the stems of ninety-eight had elongated, while only sixteen remained in the rosette condition. On the other hand, of the eighty-seven control plants one was in flower and the stems of seven had elongated.

The data indicate that injury to the stem results in a strong tendency to flower. Because of this, care must be exercised in transplanting and subsequent handling.

#### EXPERIMENTS WITH ANNUAL BEET

CONTINUOUS ILLUMINATION OF VARYING INTENSITIES FROM TIME OF GERMINATION.—Since dill, as well as a number of other long-day plants, will flower if kept on continuous day, beet was tested to see whether it required a dark period in order to bolt; that is, for the stem to elongate and to flower. Approximately 100 beet fruits were planted April 18, 1940, on the surface of the soil in flats and placed



in the greenhouse. Supplementary illumination was supplied by fluorescent Mazda lamps of the daylight type from 5:00 P.M. to 8:00 A.M., amounting to approximately 700 foot-candles at the surface of the soil. As the plants grew the reflector containing the fluorescent lamps was adjusted at higher levels. The intensity at the surface of the upper leaves, however, never fell below 200 foot-candles.

The seeds germinated rapidly and within 31 days the plants were producing flower stalks. As previously noted, dill which was growing under the same conditions required 10 days longer to produce inflorescences. Another point of contrast was that the leaves of beet grew large and succulent, whereas those of dill were small and expanded only slightly. Also the flower stalks of the beet were much taller than in dill, some of them attaining a height of over 100 cm., while those of dill averaged 10–15 cm.

Since variability of light intensity might be a factor involved in the speed with which beet responded to continuous illumination, an experiment was set up June 26, 1940, in a darkroom where the sole source of illumination was from fluorescent lamps. One set of lamps was adjusted to give 500 foot-candles at the surface of the soil, while the other gave slightly over 1000 at the same level. These lamps burned continuously and were not readjusted, so that as the plants grew in height the intensity at the leaf surface increased; the maximum attained was 1400 foot-candles. Temperature usually fluctuated about 4° C. during the day and ranged from 18° to 29° C. during the experiment.

When the beet fruits were planted, they were almost completely covered with soil and were thoroughly watered. Germination occurred a few days later. Twenty-eight days after the experiment was begun two of the plants receiving the highest intensity were developing inflorescences while the internodes of twenty-four had elongated. After 51 days the experiment under the highest intensity was terminated. By that time seventy-eight plants had bolted. The experiment under the lower light intensity was continued 25 days longer. At that time none of the plants had bolted. All grew vigorously vegetative, however, and had large deep green leaves.

#### CONTINUOUS ILLUMINATION AND NUMBER OF DAYS REQUIRED FOR INDUCTION.—

It has been reported (5) that annual beet will flower if given fifteen or twenty photoperiodic cycles each consisting of 19 hours of light and 5 hours of darkness. To determine the effect of a longer photoperiod on the number of days for induction, 220 uniform plants were divided into two equal groups and transferred from cycles consisting of 8 hours of light and 16 hours of darkness to the following treatments. One group was placed on cycles of 21 hours of light and 3 hours of darkness; the second group was transferred to continuous light. After all the plants had received eight cycles of their respective treatments, ten were transferred each day from each of the two treatments to cycles consisting of 8 hours of light and 16

hours of dark. This was continued until the last group of plants had received eighteen cycles. Approximately a month later it was found that half the plants which had received continuous illumination for 11 days had bolted. Slightly more than half the plants receiving twelve cycles, each consisting of 21 hours of light and 3 hours of dark, had bolted. The internodes of such plants were relatively short and the leaves on the stems were larger than those on the plants which received thirteen or more cycles of either continuous light or 21 hours of light and 3 hours of dark. From their appearance the stalks seemed to be more leafy than completely induced plants. Bolting in plants having received 11 days of continuous light was little more pronounced as to height attained and was only a little more rapid than in plants which received the other treatment. The data indicate that thirteen or more cycles conducive to flowering are necessary before annual beet will be as completely induced, as shown by its bolting characteristics, as plants maintained continuously on cycles consisting of 21 hours of light and 3 hours of darkness.

INTENSITY OF LIGHT REQUIRED FOR INDUCTION.—In the open greenhouse, where beet was found to be induced on continuous photoperiod and on cycles consisting of 21 hours of light and 3 hours of darkness, the light intensities varied constantly during the day from 6000 foot-candles to approximately 100, and the intensity of supplementary illumination in the various experiments ranged from 70 to 750 foot-candles. There was also considerable diurnal fluctuation in temperature. Because of these variations in light and temperature, an experiment was designed to determine the minimum constant intensity of light which would induce flowering. Early in June forty-five uniform plants were selected from the short photoperiod bench, divided into three lots, and transferred to a basement room where they could be illuminated only by fluorescent lamps. Three arrangements were made, two provided 900 foot-candles at the leaf surface, the other 700. One of the former was regulated to provide cycles consisting of 20 hours of light and 4 hours of darkness, the other burned continuously. The one at 700 foot-candles also burned continuously. Temperatures ranged from 18° to 29° C. during the experiment.

Ninety-one days after the experiment began eleven of the plants receiving continuous light of 900 foot-candles had begun to flower or had elongated. Those exposed to cycles of 20 hours of light and 4 hours of dark responded more slowly, but nine of the fifteen did flower. Of the plants receiving 700 foot-candles continuously all but three remained vegetative. In another experiment, fifteen plants were maintained under conditions of continuous light at 700 foot-candles for more than 3 months, during which time but two plants flowered.

The minimum intensity of continuous light required for flowering in beet is approximately 700 foot-candles. If the intensity were to fall much lower the plants would probably all remain vegetative. Even at 700 foot-candles the plants re-

mained green for the duration of the experiment and appeared to be not greatly different from those grown in the greenhouse continuously. Some time after the plants were placed under the fluorescent lights, however, the newly forming leaves were a darker green than those grown in natural daylight and the plant had two or three fewer leaves, but the leaves were manufacturing sufficient food materials to store starch in the base of the expanded portion.

**CRITICAL DAY LENGTH.**—To determine the critical day length of annual beet, groups of fifteen plants were placed in small dark chambers (April 15, 1940) lighted by Mazda filament lamps providing 80–100 foot-candles at the leaf surface. The photoperiod consisted of 9 hours of natural daylight, supplemented as required to provide 9, 10, 11, 12, 13, 14, and 16-hour photoperiods out of each 24 hours. Two other groups of plants were used as controls. One of these was placed on long photoperiod of about 21 hours while the other received continuous illumination.

Within 20 days all the plants having continuous illumination and those receiving cycles of 21 hours of light and 3 hours of darkness were flowering. Twenty-four days later (June 12) four plants receiving cycles of 16 hours of light and 8 hours of darkness were bolting. By July 21 the remaining eleven plants had flowered or were showing some elongation. On this date four of the fifteen plants receiving cycles of 14 hours of light and 10 hours of darkness were flowering or were elongating. There was no flowering in any of the other treatments. The data indicate that the critical day length for this variety of beet lies between 13 and 14 hours; that is, it does not flower if subjected to more than 9 or 10 hours of darkness in each 24-hour period.

Light intensity is probably an important factor in determining critical day length and in influencing the time necessary for response. On July 9, when the day length was 14 hours and 54 minutes, 120 plants were divided equally into four groups. One group received 21 hours and 37 minutes of light per day, a second 20 hours and 37 minutes, a third 19 hours and 37 minutes, and a fourth natural day length. Illumination supplemental to natural day length was provided by Mazda filament lamps. Owing to the decreasing duration of light per day, the total amount of light received at the end of the experiment was considerably less than at the beginning. Only 17 days later the plants in all three groups receiving supplementary illumination were bolting. Eleven days later nineteen out of thirty of the plants receiving natural day length were bolting.

The indication from a comparison of the speed with which beet responded to natural day length and to 9 hours of sunlight plus 7 hours of Mazda filament light is that either intensity or quality of light given after 9 hours of daylight is important in governing the rapidity with which bolting is induced.

"CUMULATIVE EFFECTS" OF EXPOSURE TO LIGHT.—There has been abundant evidence on the cumulative effect of photoperiodically inductive cycles on subsequent response to both long and short-day plants. This point is well illustrated by those plants which require more than one photoperiod of a definite length for induction. In some plants the requisite number of photoperiodic cycles for induction must be given without interruption. Biloxi soybean is one of these. Under ordinary circumstances it flowers only after exposure to four consecutive cycles consisting of 13 hours of light and 11 hours of dark. LONG (9) demonstrated the presence of cumulative effects, which endured for 24 hours, in *Xanthium*. Evidence from experiments with beet, however, indicates that the effects of ten consecutive cycles consisting of 18–20 hours of light and 6–4 hours of dark may last as long as 16 days, at which time four or five more long photoperiods consisting of 18–20 hours of light out of 24 result in flowering (5).

In order further to examine the tendency of beet to show this so-called cumulative effect of long photoperiods, a number of experiments were performed. Some of these involved the use of cycles of light and dark of equal duration, giving a total of 12 hours of light and 12 hours of dark. GARNER and ALLARD (3) found that if long-day plants were given cycles of light and dark ranging from 6 hours' light and 6 hours' dark to 1 minute light and 1 minute dark, they would respond as though on long photoperiod even though the total amount of light received was less than required for flowering if given continuously.

The outline of procedure was as follows: (a) 5 minutes of light and 5 minutes of dark; (b) 30 minutes of light and 30 minutes of dark; (c) 24 hours of light and 24 hours of dark. Other experiments involved cycles in which the light and dark periods were not of equal duration. One of these was designed to give one cycle with 8 hours of light and 16 hours of dark, followed by one cycle with 21 hours of light and 3 hours of dark; and then each cycle was repeated in order. Another similar experiment was performed in which three cycles, each consisting of 8 hours of light and 16 hours of dark, were followed by three cycles each consisting of 21 hours of light and 3 hours of dark. Flowering occurred in none of these experiments, even though the minimum duration of such continuous treatment in any instance was 2 months.

It has been reported that at least one long-day plant, dill, can be induced to flower by exposing a single leaf to conditions of long photoperiod (7). Repeated attempts have been made to cause beet to bolt by exposing a single leaf to conditions of long photoperiod, but in no instance has this resulted.

A number of experiments with short-day plants have shown that the physiological conditions involved in induction can be transmitted from one plant to another of the same species (6, 11, 12). In some of MELCHERS' work (12) involving the grafting of different species, a vegetative plant was induced to flower when it was

grafted to a different species which was flowering. If other vegetative plants could be thus induced to flower, considerable affirmative evidence could be brought to bear on the hormone theory of floral initiation.

Numerous grafts between different varieties and species were made. Among the grafts tried was one in which annual beet, of an unnamed variety closely related to biennial beet, was approach grafted, in the region of the hypocotyl, to biennial beet. Immediately after grafting the two varieties, they were placed on the long-day bench. After 3 weeks the annual beets were flowering vigorously. Although the grafts took firmly, the biennial beets remained vegetative throughout a period of 2 months, when the experiment was discontinued. All evidence from the grafting experiments indicated that if there was a flower-inducing substance transferred from one plant to another, the biennial variety still did not flower.

### Discussion

With an increase of critical data on floral initiation and vernalization processes, sufficient information is now available to permit some speculation as to the phases involved. Three of the more direct hypotheses which have arisen as a result of vernalization and photoperiodic work have been those of GREGORY and PURVIS (4), MELCHERS (12), and HAMNER (5).

It is significant that while these workers have advanced their theories independently and have used different lines of approach, their conclusions are fundamentally similar. They all agree that there is some substance or physiological condition necessary before photoperiod becomes an active agent in altering the plant's physiology sufficiently to induce it to change from the vegetative to the reproductive state. They also agree that photoperiodic cycles of light and dark can then determine the production of another substance or alter the physiological condition sufficiently to bring about the initiation of floral primordia.

HAMNER (5) has conducted a number of experiments bearing on the subject of floral initiation in two short-day plants, cocklebur and Biloxi soybean. While he is conservative in his interpretations of the substances or conditions involved, he demonstrated that conditions previous to photoperiodic induction could limit, to a certain extent, various activities which occurred during induction. Of course, this might be interpreted in terms of MELCHERS' hypothesis to indicate that the degree or amount of vernalin present had been affected previous to the photoperiodic inductive treatment.

HAMNER was chiefly concerned in his interpretation of the causes related to floral initiation and with the importance of not only the light and dark phases of photoperiodic cycles leading to induction but also of the conditions which prevailed subsequent to the imposition of such cycles or series of cycles. From his work and that of his coworkers, he suggested that there was a physiological condi-

tion or change brought about during the light period which he designated as A; others which took place in darkness were designated as B; and those which might be considered as post inductive were termed C. For brevity of reference he used the progression A, B→C to indicate the entire interrelationship.

In previous experiments involving reactions to 14 hours of light followed by 10 hours of darkness, dill remained in the rosette condition. In these experiments it was found that if the cycle was 9 hours of light and 7 hours of dark, then 1 hour of light and 7 hours of dark, the plants responded by elongating their stems. If more than 1 hour of light was given in the middle of the dark period the stems elongated at a rate almost proportional to the duration of the interposed light phase. This suggests that there is a tendency for dill to assume a physiological condition which becomes increasingly strong for the promotion of stem elongation during the photoperiod, but that during a dark period longer than the critical there is a retrogression to the condition prevailing previous to illumination. This retrogression may be slow and require the full dark period. Light may arrest it. If the dark period is interrupted, therefore, retrogression in this physiological tendency is greatly lessened and the degree to which it is halted is roughly proportional to the duration of light received during the dark period.

That there is a tendency for accumulation of small amounts of some kind of flower-forming stimulus in dill, even though it is on cycles consisting of 9 hours of light and 15 hours of dark, is indicated by the fact that after approximately 9 months of such cycles all of a group of fifty plants flowered within approximately 1 week. The tendency for darkness, in excess of the critical, to retard development of dill as well as other long-day plants may be of varying effectiveness. MURNEEK (13) points out that *Rudbeckia* may be maintained in a rosette condition on photoperiods of 12 hours or less for more than a year, provided the temperature does not become extreme. High temperature, however, will overcome the retarding effect of darkness, and this plant will then react—so far as reproductive response is concerned—as an indeterminate.

In an experiment designed to determine the critical day length of annual beet, further evidence was given for the accumulation of a flower-promoting stimulus. On continuous day beet began to show some stem elongation about 14–16 days after treatment began. Those plants receiving a 21-hour photoperiod began to bolt a few days later. It was not until 12 weeks after the experiment had begun that all the plants having cycles, each consisting of 16 hours of light and 8 hours of darkness, had bolted. After the same interval of time four of the fifteen plants receiving cycles of 14 hours of light and 10 hours of dark had bolted. While this experiment is not conclusive, it appears that over a period of time there is a slow accumulation of the residual effects of light which is slightly in excess of the

critical day length, ultimately reaching a threshold value which results in stem elongation and floral initiation.

Intensity of light was shown to play an important role in altering the physiological state of both dill and beet sufficiently to bring about flowering. In an experiment with dill it was found that when different groups of plants were exposed to different intensities of continuous light, their response in stem elongation was almost proportional, up to 300 foot-candles, to the amount of light received. With beet the plants flowered only after receiving light of 900 foot-candles for 6 weeks. Plants similarly treated, except that the light intensity was 700 foot-candles, flowered in only two cases out of fifteen.

Another environmental factor of importance in altering the response of dill to long photoperiod was temperature. If plants having cycles each consisting of 20 hours of light and 4 hours of darkness were maintained at a temperature of  $4.5^{\circ}\text{C}$ . they were only slightly responsive. The longer they received this treatment the less response they gave. From a theoretical viewpoint this would seem to indicate that higher temperatures are necessary for the efficient change of physiological conditions sufficiently to bring about stem elongation and flowering.

In general it appears that in dill—even under conditions of long photoperiod or in some instances continuous light—the greater the duration (at least beyond 1, 2, and 3 days) of such conditions as exposure to 4 foot-candles of Mazda filament light, temperatures of  $4.5^{\circ}\text{C}$ ., and intensities of red light of 50 foot-candles, the weaker the response in terms of average elongation of stems.

### Summary

1. If maintained on continuous light in the greenhouse, dill flowered in 40 days after sowing the seeds and beet in 31 days. Under conditions of relatively constant illumination at 1000–1400 foot-candles, dill required 40 days from time of planting to show some stem elongation; approximately 20 more days were required to bring about the same response under 500 foot-candles. Under similar conditions of constant illumination of high intensity, beet produced seed stalks 28 days after planting. On the other hand, beet grown under 500 foot-candles did not produce seed stalks.

2. With increasing amounts of illumination during the middle of the dark period, up to 6 hours, the average height of the dill plants was almost directly proportional to the amount of supplementary illumination received.

3. Under conditions of high light intensity, dill may be induced to flower by giving it one long photoperiod consisting of 20–21 hours of light and 4–3 hours of dark. Rapidity of stem elongation was about the same when the plants received one, two, or three long photoperiods. But if the plants received four long photoperiods, they elongated about as rapidly as those maintained on long day.

4. Dill plants previously treated with a high light intensity showed a response to continuous illumination of only 5 foot-candles if exposed to it for as little as 1 day. Those subjected to this treatment for 2 days showed some response, while those having 3-8 days of low light continuously were very slow in responding.

5. When dill was pre-treated with three short photoperiods each consisting of 9 hours of light of 4-12 foot-candles and 15 hours of darkness, and then subjected to intensities of 50-1000 foot-candles, there was a steady increase in average height with increase in intensity up to 300 foot-candles. The effect of higher intensities (500-1000 foot-candles) on rate of stem elongation was approximately the same as that produced by 200 foot-candles.

6. The effect of low temperature ( $4.5^{\circ}\text{C.}$ ) on dill plants receiving cycles consisting of 20 hours of light and 4 hours of darkness was to suppress markedly the flowering response. Those plants receiving 2 days of cold treatment attained a greater height than did any of those receiving fewer or more days of such treatment.

7. A large proportion of young dill plants elongated their stems if the roots or stems were injured. Thus injury seemingly altered the metabolism sufficiently to bring about the flowering response.

8. Red light of wave lengths greater than  $580\ \mu$  are capable of inducing the flowering response in dill when used as the sole source of illumination during the induction period.

9. Although red light of 50 foot-candles was approximately as effective in bringing about induction in dill as any intensity up to 500 foot-candles, it was not nearly so effective after 5 days of treatment as the higher intensities.

10. Annual beet did not flower if it received more than 10-11 hours of darkness in each 24.

11. Annual beet began to bolt after return to short day if it received 11 days of continuous light. The bolting of such plants, however, was no more rapid or pronounced than in those receiving cycles consisting of 21 hours of light and 3 hours of darkness.

12. Continuous illumination with 900-1000 foot-candles of light from white fluorescent Mazda lamps, under conditions of only slight temperature fluctuation, was conducive to flowering in beet. If the intensity did not rise above 700 foot-candles during the induction period, flowering did not occur.

13. Annual beet did not flower if subjected to the following cycles of light and dark: (a) 5 minutes of light and 5 minutes of dark (55 days); (b) 30 minutes of light and 30 minutes of dark (55 days); (c) 24 hours of light and 24 hours of dark (117 days); (d) one short photoperiod of 8 hours of light, 16 hours dark and one long photoperiod of 21 hours of light, 3 hours dark (117 days); (e) three short



photoperiods of 8 hours of light, 16 hours dark and three long photoperiods of 21 hours of light, 3 hours dark (117 days).

14. Exposure of a single leaf of beet to cycles of 20 hours of light and 4 hours of darkness did not result in the plant flowering.

The writer wishes to express his appreciation of the helpful suggestions and continued interest of Dr. K. C. HAMNER during the course of these experiments.

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# EFFECTS OF ROOT-GROWTH HORMONES ON THE MERISTEM OF EXCISED PEA ROOTS<sup>1</sup>

FREDRICK T. ADDICOTT

(WITH SIX FIGURES)

## Introduction

Roots of pea, radish, flax, and tomato, whose nutrition has been studied by experiments with cultures in vitro, require vitamin B<sub>1</sub> as an accessory growth factor (4, 7, 6). For maximum growth certain of these roots (pea, tomato, radish) require in addition nicotinic acid (2, 6). Since in the plant vitamin B<sub>1</sub> and nicotinic acid have all the characteristics of hormones, it seemed of interest to investigate their histological effects. This paper presents observations of the meristems of excised pea roots cultured under conditions of deficiency of vitamin B<sub>1</sub> or of nicotinic acid. The anatomical effects of vitamin B<sub>1</sub> deficiency on isolated pea roots has been described in an earlier paper (1). It was found that in the absence of vitamin B<sub>1</sub> from the culture medium, cell divisions in the apical meristem of the root cease. The roots used in the earlier investigations, however, were cultured in medium containing no nicotinic acid. Such roots suffer primarily from deficiency of vitamin B<sub>1</sub>, but the symptoms observed might have been attributable in part to deficiency of nicotinic acid. It seemed advisable, therefore, to examine roots in which these two deficiencies were present independently.

Roots of *Pisum sativum*, variety Perfection, were cultured by methods previously described (5, 3) for five weekly transfers. The experiment proper was not started until after the fifth transfer, in order to insure that the roots were well established. After this time roots receiving both vitamin B<sub>1</sub> and nicotinic acid in addition to salts maintain a uniform appearance and growth rate in all succeeding transfers. Two hundred and fifty roots, which had averaged 75.6 mm. of growth during the fifth week, were selected. These were divided into three lots: one received vitamin B<sub>1</sub> as the only accessory growth substance; a second received only nicotinic acid; and the third, the control group, received both substances. Growth was measured at the time of each weekly transfer, and as the effects of the deficiencies became apparent, samples of the roots were fixed for sectioning and further study. The material was fixed in Navashin's solution and always at noon, in order to eliminate variation in the number of cell divisions which might result

<sup>1</sup> Work carried out with assistance from the Works Progress Administration, Official Project 665-07-3-83, W.P. L-9809.

from fixation at different stages in the cycle of diurnal fluctuation of mitosis. The root tips were dehydrated with tertiary butyl alcohol and sectioned in paraffin. Sections were stained with iron-haematoxylin.

### Observations

The growth rate of roots receiving only vitamin B<sub>1</sub> or only nicotinic acid declines and in the course of a few weeks reaches zero. In the absence of vitamin B<sub>1</sub> growth falls off more rapidly than in the absence of nicotinic acid (table 1). This accounts for the fact that vitamin B<sub>1</sub> was the first accessory growth factor discovered for excised pea roots. Figures 1-3 show the habit of the roots in culture and that deficiency of vitamin B<sub>1</sub> reduces growth rate to a greater extent than does deficiency of nicotinic acid. Roots lacking nicotinic acid become thin, particu-

TABLE 1  
GROWTH OF PEA ROOTS IN ABSENCE OF VITAMIN B<sub>1</sub> OR NICOTINIC ACID

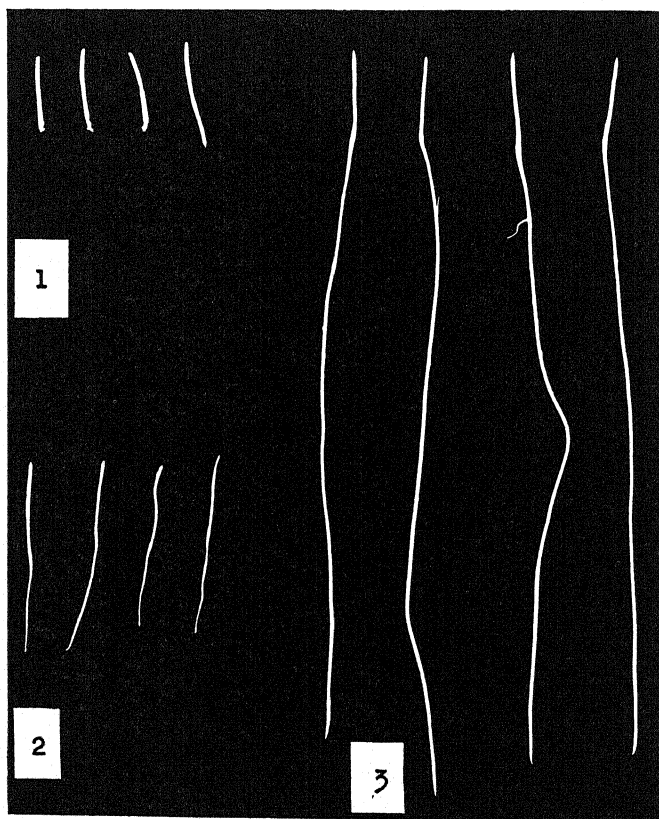
ACCESSORY SUBSTANCES IN MEDIUM	GROWTH RATE IN MM. PER WEEK				
	5TH*	6TH	7TH	8TH	9TH
Vitamin B <sub>1</sub> plus nicotinic acid.	75.6	81.0	80.3	78.2	82.4
Vitamin B <sub>1</sub> alone.....		77.4	55.9	32.2	17.8
Nicotinic acid alone.....		79.1	59.8	22.2	8.2

\* Roots cultured in medium containing vitamin B<sub>1</sub> and nicotinic acid during the first 5 weeks.

larly toward the apex; those lacking vitamin B<sub>1</sub> remain thick and frequently show irregular swellings.

A preliminary examination of the sections showed that roots deficient in vitamin B<sub>1</sub> and those deficient in nicotinic acid differed not only from the controls but also from each other. The length of the meristem and the number of dividing cells it contained, as well as the width of the meristem and the number of columns of cells, were all affected by both deficiencies. Both deficiencies also affected cell elongation to some degree. The formation of starch grains and the deposition of cell-wall materials appeared to be unaffected, at least during the early stages. In order to obtain a quantitative basis for the comparison of the roots in this experiment, measurements were made of the affected anatomical features just mentioned. For this purpose median longitudinal sections were used, and counts were made from the two sections nearest the midline of each root. The results are summarized in table 2. The measurements show that in the case of both deficiencies the decline in growth rate is accompanied by a decrease in the length of the

meristem as well as in the number of its cell divisions. In 4 weeks the growth rate of roots deficient in nicotinic acid drops to about 20 per cent of that of the controls (table 1), while the meristem becomes shorter in nearly the same degree and the number of cell divisions in the meristem falls to 40 per cent of the number of the control. In the same period vitamin B<sub>1</sub>-deficient roots drop in growth rate to



FIGS. 1-3.—Shadow photographs of roots at end of ninth weekly transfer: Fig. 1, from medium lacking vitamin B<sub>1</sub>; fig. 2, from medium lacking nicotinic acid; fig. 3, control from medium containing both growth substances.

10 per cent of the rate of the controls, while the meristem becomes reduced to about 15 per cent of the length of the controls and the number of cell divisions also decreases in about the same proportion.

It is also evident from examination and measurement of the more proximal regions of the sections that the cells of both types of deficient roots do not elongate to so great an extent as do those of the control roots which received both growth substances. Both types show elongation to only about 60 per cent of the length

of the controls. The measurements were made from cells 5-6 mm. from the apex of the roots. These portions of the different roots are not strictly comparable. The deficient roots were growing at a much slower rate, thus cells 5 mm. from the apex had had more time in which to elongate than had cells the same distance from the apex of the rapidly growing roots. It appears probable, therefore, that if fully mature regions were compared, the cells of deficient roots would be found to be considerably less than 60 per cent of the length of cells in roots receiving both vitamin B<sub>1</sub> and nicotinic acid.

TABLE 2  
MEASUREMENTS TAKEN FROM SECTIONS OF PEA ROOTS DEFICIENT  
IN VITAMIN B<sub>1</sub> OR NICOTINIC ACID

ACCESSORY GROWTH SUBSTANCES IN MEDIUM	WEEK OF FIXATION	WIDTH OF MERISTEM*			LENGTH OF MERISTEM† (mm.)	NO. OF CELL DI- VISIONS IN MERISTEM‡	LENGTH OF CORTICAL CELLS§ (mm.)
		IN MM.	IN TERMS OF CELLS	AVERAGE CELL WIDTH (mm.)			
Vitamin B <sub>1</sub> and nicotinic acid (controls) . . . . .	8th and 9th	0.392	37.2	0.0106	1.50	11.9	14.3
Vitamin B <sub>1</sub> alone . . . . .	{ 8th 9th	0.310	36.3	0.0085	0.87	11.9	10.3
		0.260	32.3	0.0081	0.40	4.6	8.0
Nicotinic acid alone . . . . .	{ 8th 9th	0.384	37.9	0.0101	0.81	6.9	9.3
		0.359	37.0	0.0097	0.20	1.7	8.9

\* Measurements of width taken at proximal end of meristem, at point where cell divisions cease.

† From tip of meristem to last observable mitotic figure that was part of apical meristem.

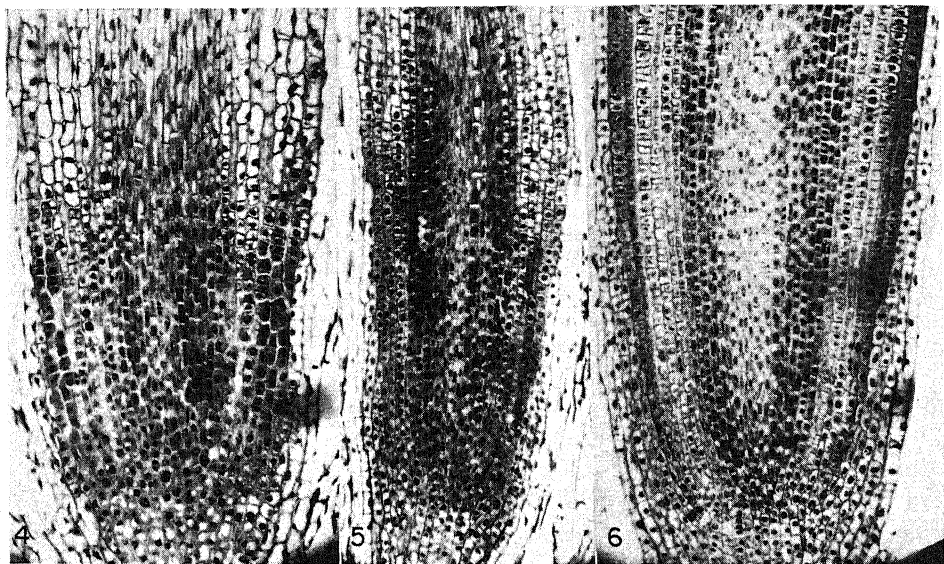
‡ These figures include only the number of cells in metaphase or anaphase to be found in median longitudinal sections. They are a measure of the number of cell divisions in the root.

§ Longest cortical cells in the region 5-6 mm. from tip measured.

The meristems of the deficient roots differ from each other also in diameter (figs. 1-6). Nicotinic acid-deficient roots averaged 65 per cent of the diameter of the controls, while vitamin B<sub>1</sub>-deficient roots averaged slightly over 90 per cent of the controls. The diameter was also measured in terms of cells. All measurements were made at the proximal portion of the meristem, where cell divisions cease. The thinness of the nicotinic acid-deficient roots was correlated with the smaller size of their cells and the fact that such roots contain fewer columns of cells than do the control roots. The cells of the nicotinic acid-deficient roots averaged about 75 per cent of the diameter of similar cells of the control roots, and the sections of the deficient roots contained only 85 per cent as many columns of cells as did the controls. The thickness of the meristem of vitamin B<sub>1</sub>-deficient roots is only slightly less than that of the controls. The number of columns of cells in these meristems is the same as that of the controls, but the average diameter of the cells, being about 90 per cent of the controls, accounts for the slightly thinner

overall diameter of the vitamin B<sub>1</sub>-deficient roots. As noted, the nicotinic acid-deficient roots remain smooth while vitamin B<sub>1</sub>-deficient roots often develop irregular swellings. No detailed examination was made of these swellings, but from limited observations it appeared that they are due to a radial enlargement of groups of cortical cells.

Roots in the most advanced stages of deficiency studied, that is, those cultivated in deficient medium for 4 weeks, showed considerable starch deposition as well as every indication of active cell-wall formation. Starch grains are conspicu-



FIGS. 4-6.—Sections of roots made at end of ninth weekly transfer: Fig. 4, cultured in medium lacking vitamin B<sub>1</sub>; fig. 5, in medium lacking nicotinic acid; fig. 6, control cultured in medium containing both growth substances.

ous in the region of the meristem, especially in the vacuolated cells of the epidermis and root cap. They are rarely seen in similar regions of the actively growing control roots. Likewise the cell walls of both types of deficient roots appear to be well formed and heavy, especially in the root cap and peripheral regions of the meristem. In the deficient roots also there is a strong tendency for the root cap to adhere to the tip. It is doubtful whether root-cap formation is more extensive in the deficient roots, since the controls regularly form large masses of cap tissue. In the latter case, however, the caps rarely remain attached to the roots after the process of fixation and dehydration. Figures 4-6 show the topography of the affected roots, their relative diameters, and the extent of the meristematic regions after 4 weeks in deficient medium. Portions of the root cap can be seen adhering to the roots cultured in deficient media.

### Summary

1. Deficiencies of the root-growth hormones vitamin B<sub>1</sub> and nicotinic acid in cultures of excised pea roots have visible effects in the meristem.

2. The deficiency of either substance results in a decrease in the growth rate and eventual complete cessation of growth. This is accompanied by reduction in the length of the meristem, decrease in the number of cell divisions in the meristem, as well as reduction of the total length attained by the cells as they mature.

3. Roots deficient in nicotinic acid become thin. This is accompanied by reduction of the diameter of the root cells and of the number of columns of cells in the roots.

4. The meristems of roots deficient in vitamin B<sub>1</sub> become only slightly smaller in diameter in the course of 4 weeks, but their growth rate declines more rapidly than does that of roots deficient in nicotinic acid. The mature portions of these roots develop irregular thickenings.

Most of this work was done at the California Institute of Technology, and the writer is indebted to Dr. JAMES BONNER and Dr. F. W. WENT for helpful suggestions during the course of the investigation.

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# GROWTH OF SOME RANGE GRASSES IN REDUCED LIGHT INTENSITIES AT CHEYENNE, WYOMING

H. M. BENEDICT

(WITH FOUR FIGURES)

## Introduction

Several investigations (10, 4, 1, 3, 9) have been carried out on the growth of plants in full sunlight and shade, but only two of them—one dealing with sunflowers (5) and the other with trees (8)—have been conducted in regions where the intensity of full sunlight is greater than 10,000 foot-candles. At Cheyenne, Wyoming, sunlight intensities as high as 14,000 foot-candles (as measured by a Weston meter) have been recorded, and readings of 12,000 are common on clear days. Repeated readings taken at random between 9:00 A.M. and 3:00 P.M. from June to September, inclusive, have almost always shown the intensity to be above 10,000 foot-candles on cloudless days. Since such intensities are higher than reported in some other sections of the country, the question was raised as to whether or not these intensities were too high for the best growth of some of the range grasses.

This paper presents the results obtained when plants of crested wheat grass (*Agropyron cristatum* (L.) Gaertn.), bluestem (*A. smithii* Rydb.), and blue grama (*Bouteloua gracilis* (H.B.K.) Lag.) were grown in full sunlight and under shades transmitting various percentages of full sunlight but not changing the day length.

## Methods

Seed of the various species, gathered the previous year from plants growing in the grass nursery at Cheyenne, were planted in boxes 1 × 1 × 1 feet, filled with soil. After the seed had germinated the seedlings were thinned to twenty-four per box and grown until the end of the experiment. The soil was then carefully washed from the plants and their dry weight determined by drying to a constant weight at 100° C. In 1938 the seed was planted June 7 and the plants harvested October 1. In 1939 the seed was planted June 14 and the plants harvested October 3.

Four intensities were used in 1938: 100 per cent, 57 per cent, 42 per cent, and 28 per cent of full sunlight. The shaded plants were grown in a lathhouse under none, one, and two layers of ordinary wire window screen. The shaded plants were about 20 feet from the unshaded ones, but this difference in location probably had no appreciable effect on their growth.

Because of the wide gap between 100 per cent and 57 per cent sunlight, and be-

cause it was possible that the wide bands of full light and shade passing across the leaves of the plants growing in the lathhouse might have had some effect on the results, the experiment was repeated in the summer of 1939, with the following modifications. In the 1939 series the plants were grown in the open in full sunlight and in the shade cast by one and two layers of window screen or in 100 per cent, 70 per cent, and 50 per cent of sunlight intensity, respectively. In this series the different treatments were randomized, thus eliminating the possibility of any positional effect being responsible for the differences occurring in the treatments.

In order to shade the plants with the window screen, wooden frames  $4 \times 4 \times 4$  feet were constructed and one or two layers of screening secured to four of the six faces. These screened frames were then placed over groups of nine boxes, three boxes of each species, one open side on the ground and one facing north. Thus the plants were shaded from above and on the east, south, and west sides, leaving the north side open, so that access could be had to the plants. Except for short periods in early morning and late evening during the early part of the summer, this kept them shaded at all times from the sun.

The values given for the percentages of full sunlight transmitted by the various screens are those obtained when the screens were placed at right angles to the direction of the sun's rays. GARNER and ALLARD (7) have pointed out that the percentage transmission of screens of the type used in these experiments varies with the angle at which the light strikes the screen, the greatest transmission occurring when the light is traveling at right angles to the screen. The relative light intensities in the various treatments were checked once a week with the Weston meter.

In both years the soil used was carefully mixed to insure uniformity among treatments. In 1938 the boxes were all filled to the same level, so that approximately the same amount of soil was placed in each flat. In 1939 the same weighed amount was placed in each flat. The flats were watered whenever they required it. Of course the plants in the reduced light intensity required watering less often than those in full sunlight. The flats of the 1939 series were weighed occasionally during the summer, and at no time was there any difference in the weights of the flats in the various intensities. Since the same amount of soil was placed in each box at the beginning of the experiment, and assuming the weights of the plants to be negligible, evidently the amount of moisture available to the plants was approximately the same in all treatments. While these weights were not taken in 1938, the same method of watering was employed, and presumably there was no appreciable difference during that year. In neither year did the plants in any treatment wilt.

It was never possible to demonstrate a difference in air temperature in any of the treatments in either year. Soil temperatures taken 2 inches beneath the surface were  $1-2^{\circ}$  C. higher in the flats exposed to full sunlight than in those that were

shaded. While these differences in soil temperature were consistent, they were not great enough to account for the differences obtained between the plants in the different treatments.

### Results

The results are the means of nine replications for 1938 data and six for 1939 data, each replication consisting of twenty-four plants. Thus in 1938 there were 216 and in 1939, 144 plants of each species in each treatment.

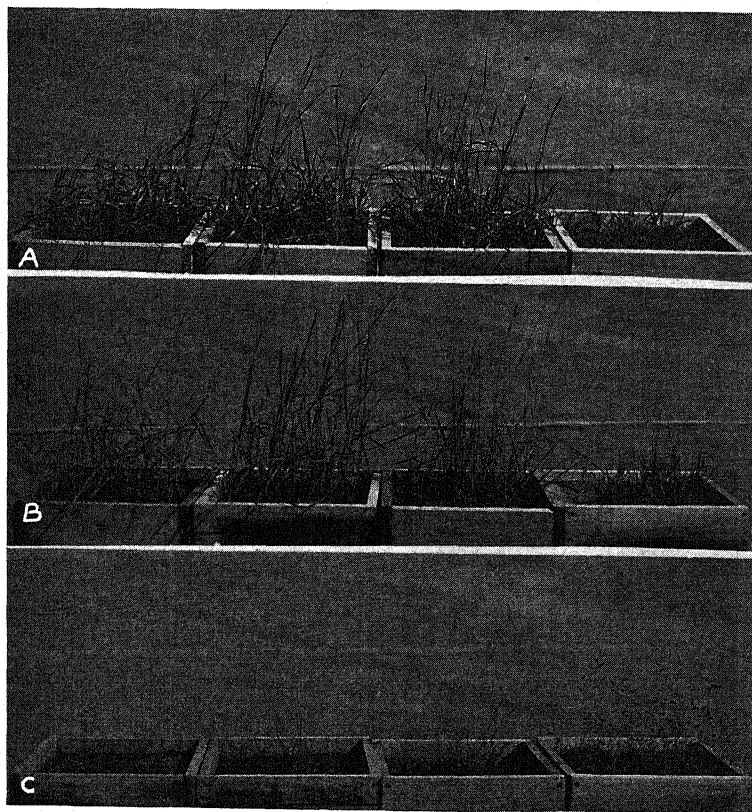
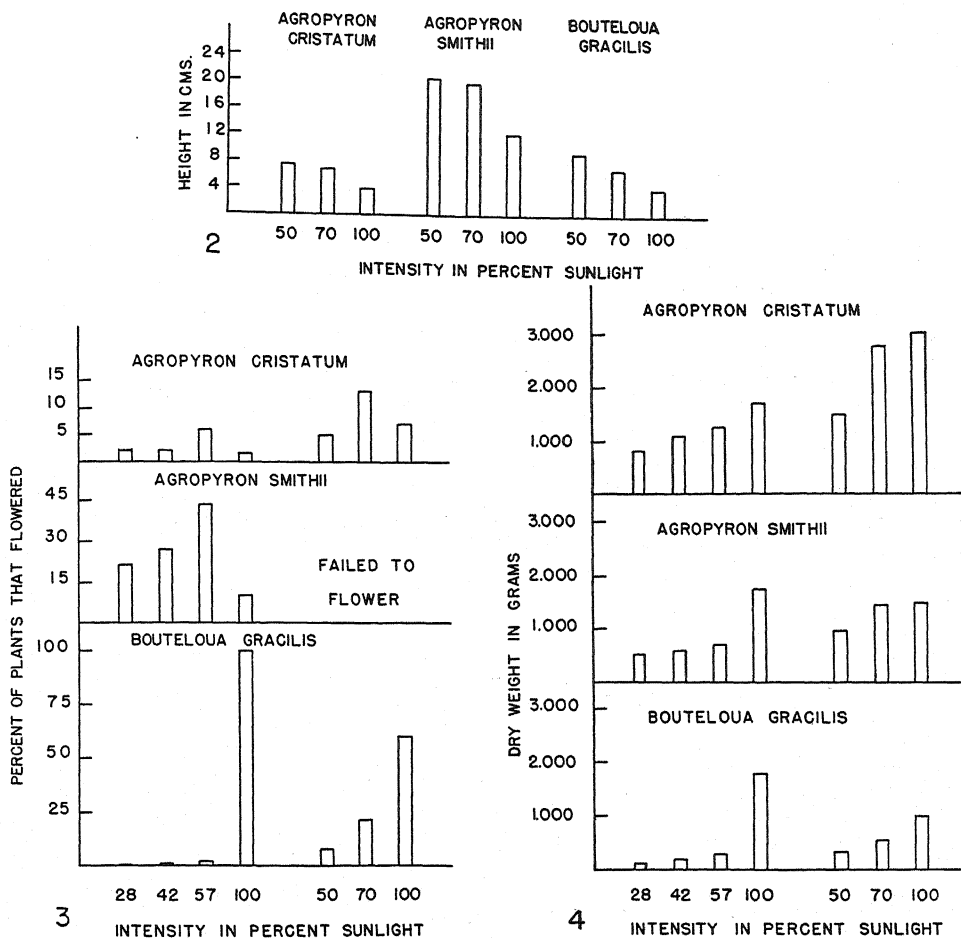


FIG. 1.—Plants grown in (left to right) 28 per cent, 42 per cent, 57 per cent, and 100 per cent sunlight in 1938: A, *Agropyron cristatum*; B, *A. smithii*; C, *Bouteloua gracilis*.

Representative plants in the different treatments at the end of the summer of 1938 are shown in figure 1. The shaded plants elongated and became spindly, while the unshaded plants were short and assumed an almost rosette type of vegetative growth. Photographs of the plants grown in 1939 are not shown, but the same type of growth was noted in both the shaded and unshaded plants.

The heights of the plants were not recorded in 1938, but figure 1 indicates that

as the light intensity was reduced the height of the vegetative parts of the plants of the three species increased to a maximum and then decreased, the greatest height being reached at 42 per cent full sunlight. Plants grown in 70 per cent and



FIGS. 2-4.—Fig. 2, height of plants grown in different light intensities in 1939. Fig. 3, number of plants that bloomed in different light intensities in 1938 (left) and 1939 (right). Fig. 4, dry weights of entire plants grown in different light intensities in 1938 (left) and 1939 (right).

50 per cent sunlight were always taller than those grown in 100 per cent (fig. 2), but the tallest plants of all species were in 50 per cent sunlight. These figures are in general agreement with the observations made on the heights of the plants grown in 1938.

Counts were made of the number of plants that flowered in the various treatments, and the results, calculated in percentage of the total number of plants in

each treatment, are shown in figure 3. A greater percentage of plants of *Bouteloua gracilis* and a smaller percentage of *Agropyron cristatum* and *A. smithii* flowered in 100 per cent sunlight than flowered in the reduced light intensities. Also the seed heads of *A. cristatum* and *A. smithii* were much larger on the plants growing in the shade than on those in full sunlight, regardless of the percentage of plants that flowered. Plants of *B. gracilis*, however, seemed to have the largest seed heads when grown in 100 per cent sunlight. No measurements were made on the actual

TABLE 1  
DRY WEIGHTS IN GRAMS AND ROOT-TOP RATIOS OF PLANTS  
GROWN IN DIFFERENT LIGHT INTENSITIES

PART OF PLANT AND SPECIES	LIGHT INTENSITY AS PERCENTAGE OF FULL SUNLIGHT						
	1938				1939		
	28	42	57	100	50	70	100
Roots:							
Agropyron cristatum...	0.410	0.627	0.801	0.945	0.783	1.312	1.477
Agropyron smithii.....	0.220	0.240	0.310	1.075	0.520	0.902	0.989
Bouteloua gracilis.....	0.046	0.096	0.137	0.733	0.133	0.231	0.360
Tops:							
Agropyron cristatum...	0.397	0.460	0.450	0.801	0.701	1.457	1.529
Agropyron smithii.....	0.317	0.325	0.397	0.651	0.434	0.529	0.482
Bouteloua gracilis.....	0.052	0.097	0.157	1.050	0.200	0.327	0.641
Total plant:							
Agropyron cristatum...	0.807	1.087	1.251	1.746	1.484	2.769	3.006
Agropyron smithii.....	0.537	0.565	0.707	1.726	0.954	1.431	1.471
Bouteloua gracilis.....	0.098	0.193	0.294	1.783	0.333	0.558	1.001
Root-top ratio:							
Agropyron cristatum...	1.038	1.384	1.773	1.309	1.080	0.940	0.973
Agropyron smithii.....	0.657	0.741	0.811	1.761	1.273	1.753	2.162
Bouteloua gracilis.....	0.921	1.094	0.960	0.803	0.687	0.732	0.560

size of the heads, but some idea of the differences may be seen from the plants of *A. cristatum* and *B. gracilis* in figure 1.

In 1939 the seed of *A. smithii* was extremely slow in germinating, with the result that the plants had made almost no growth by July 15. Unpublished data (2) have shown this to be a long-day plant (6), and it may be that this species failed to flower because by the time the plants were large enough to be responsive to the photoperiodic stimulus, the days long enough to induce flowering had passed.

The dry weights of the plants in the various treatments are shown in table 1 and in figure 4. In general these results indicate that these species make their best growth in greater than 70 per cent of the full sunlight at Cheyenne, and that as the light intensity is decreased, at least below 70 per cent sunlight, the dry weights of

the plants decrease. They also show that reductions in light intensity are accompanied by a greater decrease in growth of *B. gracilis* than of *A. cristatum* and *A. smithii*.

The root-top ratios obtained from the dry weights as shown in table 1 indicate that reductions in the intensity of full sunlight by certain amounts may cause a smaller decrease in the growth of the roots than of the tops of *A. cristatum* and *B. gracilis*. They also show that any of the intensities used in these experiments cause a greater decrease in the growth of the roots than of the tops of *A. smithii*.

The ratios presented are not those that would be obtained by dividing the average weight of the roots by the average weight of the tops, but are the means of nine or six ratios (depending on the year) yielded by the individual replications.

### Discussion

The increase in height and the decrease in dry weight of the plants grown in the shade as compared with those grown in full sunlight are in general agreement with the results obtained by others, as reviewed by SHIRLEY (10) and BURKHOLDER (4). However, the dry weights of the plants of *Agropyron cristatum* and *A. smithii* in 100 per cent sunlight were not much greater (less in the case of the tops of *A. smithii*) than in 70 per cent sunlight. This indicates the possibility that in 80 per cent or 90 per cent sunlight the dry weights of at least two species might be equal to if not greater than those in 100 per cent sunlight.

The marked results of the shading in increasing the number of plants of *A. cristatum* and *A. smithii* that flowered indicate that, in the vicinity where these experiments were carried out, 100 per cent sunlight is so intense as to be unfavorable for the flowering of these species, at least in the first year of their growth. This may partly explain why plants of these two species flower so sparsely in field and nursery plantings during their first growing season. On the other hand, the great decrease in the number of flowering plants of *B. gracilis* in the reduced light intensities seems to show that 100 per cent sunlight is the most favorable intensity for the blooming of this species. Field and nursery plantings of this species usually flower abundantly during their first year.

The difference in blooming response and the small reduction in dry weight in the shade of plants of *A. cristatum* and *A. smithii* as compared to plants of *B. gracilis* cannot be explained on the basis of the native habitats of the species, since all three normally occur and are grown in dry plains exposed to the full intensity of the sun.

The similarity in response of the plants in the reduced light intensities in 1938 and 1939 seems to indicate that there is little if any difference between the type of shade cast by wide strips of opaque material and the type cast by window screen, as far as its effect on plant growth is concerned.

In some work on *A. smithii* and *B. gracilis* to be published elsewhere (2), and in some unpublished data on *A. cristatum*, it is shown that plants of these species grown in the greenhouse under the same temperature conditions during the winter in days lengthened by means of incandescent lamps were always much taller than plants grown in the normal or short day lengths. It is also noted that plants of *A. cristatum* and *A. smithii* bloomed only in the long days, and plants of *B. gracilis*—while they would bloom in either day length—were always slower to come into flower in the long day. Throughout these shading experiments similarities in growth form and blooming response were noted between plants growing in the reduced light intensities and in the artificially lengthened days of the winter and between plants growing in full sunlight and in the short days of the winter season. Whether or not these similarities are coincidental is not known.

### Summary

1. Plants of *Agropyron cristatum*, *A. smithii*, and *Bouteloua gracilis* were grown at Cheyenne, Wyoming, in 100 per cent, 57 per cent, 42 per cent, and 28 per cent sunlight in 1938 and in 100 per cent, 70 per cent, and 50 per cent sunlight in 1939, and the height, dry weight, and number of plants that flowered recorded.
2. The plants grown in the shade were taller than those grown in full sunlight but had a smaller dry weight.
3. Reducing the light intensity to a certain extent increased the number of plants of *A. cristatum* and *A. smithii* which flowered but greatly decreased the number of plants of *B. gracilis* which flowered.
4. Using the dry weight as a measure of growth, the results indicate that these species make their best growth in full sunlight; but that full sunlight, while favoring the flowering of *B. gracilis*, is so intense as to retard the flowering of *A. cristatum* and *A. smithii*.

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USE OF FROZEN VACUUM-DRIED MATERIAL IN AUXIN AND  
OTHER CHEMICAL ANALYSES OF PLANT ORGANS:  
ITS EXTRACTION WITH DRY ETHER<sup>1</sup>

CONTRIBUTIONS FROM THE HULL BOTANICAL LABORATORY 522

GEORGE K. K. LINK, VIRGINIA EGGERS, AND JAMES E. MOULTON

(WITH ONE FIGURE)

Standardized and quantitative methods of extraction of auxins are greatly needed to foster studies of the presence and role of auxins in normal and healthy, and in abnormal and pathic, growth in plants.

In current practice each test proceeds from a sample of fresh plant material placed either in ether, alcohol, or water, alone or in a series of one or more of these solvents (3, 20). In general, grinding or even moderate division of the material is avoided to escape the destructive action of oxidative enzymes on the auxins. It is likely that the variation in chemical constitution known to occur in plant organs or tissues from hour to hour, day to day, and growth phase to growth phase, also holds for the auxin contents of different samples of the "same" material. It seemed advisable, therefore, to devise methods which permit collection at one moment of a large sample of fresh material which might then be drawn on for identical subsamples. Such a method would require a thoroughly homogeneous sample, which could be attained only by grinding to fine powder and careful sifting. It would eliminate one of the many variables which hamper current procedures of auxologists. AVERY (1, 2) has approached this method by using air-dry seed of corn, which he grinds before extraction. SKOOG and THIMANN (17) have used ground samples previously dried by heat.

Current procedure also is based on the report and assumption (14) that a single extraction of an intact plant organ with ether, alcohol, or chloroform quantitatively removes the auxins. LINSER (12) reports that this is not the case for spinach, and AVERY (2) reports the same for corn endosperm; GUSTAFSON (7) for tomato and other plants, and SKOOG and THIMANN (17) for *Lemna* and other plants. We have made the same observation for each of the materials under study: roots and nodules of kidney bean, soybean, garden pea; leaves of cabbage and the aphids *Myzus persicae* and *Brevicoryne brassicae* which feed on them; hypocotyl of tomato and the hypocotyledonary galls incited in it by experimental inoculation with *Phytophthora tumefaciens*; the leaves and tassels of corn as well as the smut galls

<sup>1</sup> This work was aided in part by a grant from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago. It also was aided by student help provided by the N.Y.A.

incited in them by experimental inoculation with *Ustilago zaeae*, and the aphid *Aphis maidis* which feeds on them.<sup>2</sup> The findings suggest that the auxins are progressively liberated from plant tissues, and that water might play a role catalytic or otherwise in this process. It seemed advisable, therefore, to test the behavior of really dry materials in the presence of really dry solvents.

Current practice also proceeds from the observation (19) that oxidative enzymes of plants rapidly destroy auxins. For this reason fresh plant materials generally are placed as whole as possible into peroxide-free ether (3) or other solvents. This suggested that drying by heat should be avoided if possible. The alternative of arresting enzyme activity and breaking up the material by freezing, tried by DU BUY (4) in chlorophyll-containing tissues, has not come into general use because it is troublesome, and because—unless special care is taken to avoid thawing in air—the material is even more subject to destructive enzyme activity than before its injury by freezing.

#### Apparatus and methods

Seeking a method which would attain the objective of a large dry sample without much enzymatic and other chemical alteration, the senior writer recollected the class lectures given by Professor F. C. KOCH between 1910–1916 in a course on tissue analysis at the University of Chicago. In these he called attention to SHACKELL's (16) method of freezing animal tissues (solid and fluid) and then dehydrating them in vacuo, and pointed out that this would be the ideal method of preparing biological tissues for analysis, if the technical difficulties of rapid freezing and drying in vacuo could be mastered.

During a consultation with Professor KOCH in 1940 on this topic he called attention to the fact that the technical difficulties of freezing and dehydrating in vacuum had been overcome to such an extent that the method had come into commercial use by firms which process biological products, notably seric and endocrinic, from tissues. This advance is largely the result of the investigations of FLOSDORF and others working in collaboration with MUDD (5, 6, 13). Their papers contain a historical statement of the development of the process and many technical details about apparatus and techniques. TAYLOR and BEARD (18) recently described an inexpensive modification of FLOSDORF and MUDD's latest model (6) of the apparatus.

This method of preparing biological materials involves a minimum of chemical change in them. Only certain proteins and enzymes seem to be altered by the dehydration. So long as the material is solidly frozen, enzymatic activity is virtually absent. Since evaporation of the ice in the vacuum cools the material, this remains cold until dry, with the result that the enzymes are then inactive

<sup>2</sup> The work on corn, corn aphid, corn smut, and *Ustilago zaeae* is being carried out by J. E. MOULTON.

because of lack of water. Unlike tissues dried by heat or chemical reagents, these avidly take up as much fluid as they lost, and hence are called lyophile.

By this method material of legume roots and nodules, of tomato hypocotyl and gall, of corn leaf, corn smut galls, and of the fungus *Ustilago zeae* has been prepared, using the apparatus of Professor KOCH and THOMAS F. GALLAGHER of the Department of Biochemistry. Based on these experiences and in consultations with these colleagues, to whom we are greatly indebted for many courtesies, a modification of their apparatus has been devised which is suited to the handling of plant materials, which usually are more bulky than animal tissues.

The apparatus differs from some others in current use in that the distillate from the frozen material is not adsorbed by a desiccant in a trap; instead it is collected in a freezing trap and is recoverable after thawing and thus available for analysis and experimental use. Experience with each of the plant materials prepared in the apparatus shows that the distillate is not pure water; it is slightly turbid and has the odor characteristic of the material dried. Drying by heat, or drying by the recent cryochem method of FLOSDORF and MUDD (6), apparently loses some of the constituents, and consequently a complete compositional picture of the material cannot be constructed from analysis of the dried residue. In all likelihood the activity of such material also is not the same as that of fresh material.

The apparatus (fig. 1) is made of heavy pyrex glass<sup>3</sup> with interchangeable ground-glass connections. Since the average free path of the molecules in the line is an important factor in the evacuation and drying, a large and uniform passage was attained by using 34-mm. (inside diameter) tubing with 40/50 interchangeable connections.

The essential parts are pear-shaped pyrex flasks (A) with 40/50 male connections. The material may be frozen directly in such flasks. The male connection avoids use of vacuum grease inside the neck of the flask and possible contamination of the sample. The connection of each flask fits into a 40/50 female connection at the end of one of two short arms which project at an angle of 80° from the upper part of the freezing trap (B). This trap is 100 mm. in diameter and 23 cm. long below the arms, with a capacity of 1800 cc. of fluid. The arms are set at an angle of 80° to give maximum surface exposure to the material in the pear-shaped flask (500 cc. or 1000 cc.). The larger the flask, the thinner the layer of a given amount of material, and hence the more rapid the drying. The arms, 50 mm. long, are as short as possible to minimize the path of the water molecules and just to clear the outer edge of the container (C), in which the trap is set when in use. Container C is a pyrex 1-gallon food jar (Jumbo).<sup>4</sup> After the trap (B) is sus-

<sup>3</sup> Made for this work by the Scientific Glass Apparatus Co. of Bloomfield, N.J.

<sup>4</sup> Jar no. 8642 or no. 820 of the American Thermos Bottle Co. is recommended. The former is more highly evacuated than the latter and is more expensive. We are using the latter (Jumbo) with inside dimension of  $5\frac{7}{8} \times 11$  inches.

pended in this container, crushed dry ice ( $\text{CO}_2$ ) and the methyl ether of ethylene glycol<sup>5</sup> are slowly packed around it up to the rim. This mixture, which is also used for freezing the material, gives temperatures between  $-78^\circ$  and  $-80^\circ$  C. It is less expensive and more pleasant to work with than acetone. Traps *B* and containers *C* can be set up in series up to the capacity of the pump system. If additional freezing traps are used, these ( $B_1$ ,  $B_2$ , etc.) differ only in that each has an inlet arm (*i*) for connecting to the line. The large trap obviates interruption of the desiccation when large samples are being dried, and the large thermos container (*C*) reduces the time required in restocking with dry ice. A late evening stoking will carry the apparatus through the night and well into the late morning.

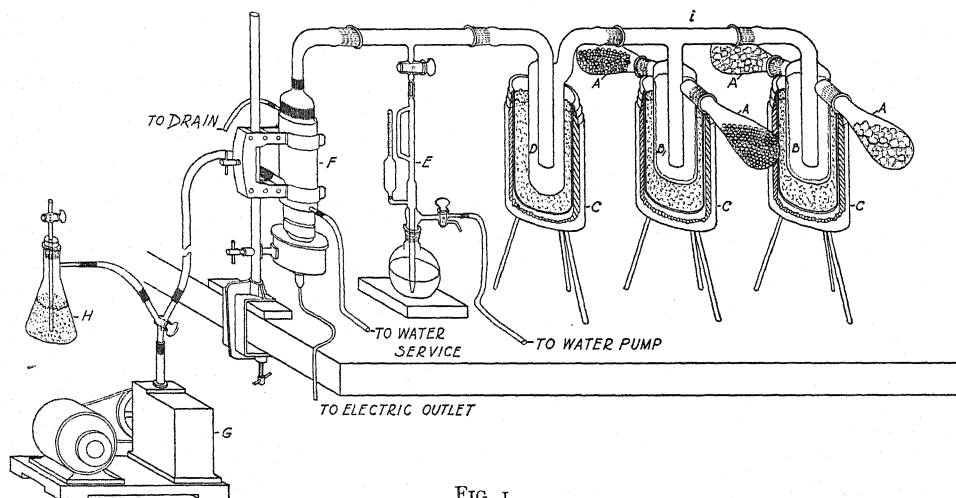


FIG. 1

Beyond the last side arm trap is a smaller safety freezing trap (*D*). This trap, 75 mm. in diameter and 23 cm. long, is set in a cellosolve dry-ice bath in a thermos jar. It ensures trapping of any water vapor which got by the other traps, and also protects these traps from the mercury of the gauge and pumps.

Beyond the safety trap (*D*) is a MacLeod mercury gauge (*E*) and beyond this a Cenco metal mercury vapor diffusion pump (Super Vac) (*F*) which is fitted to the 34-mm. pyrex line with an adapter by means of Cenco pure para rubber tape. This pump is capable of generating a vacuum of  $10^{-6}$  mm. at a speed of 7 liters per second. Such is not attainable of course in a line containing water vapor. Beyond the mercury pump is a High Vac oil pump (*G*) which serves as forepump. For best results, the line between this and the Super Vac mercury pump consists of a pyrex glass rather than of rubber tubing. Air is admitted to the system through a chemical desiccant bottle (*H*) when the vacuum is to be broken. The maximum capacity

<sup>5</sup> Sold as Cellosolve by the Union Carbide and Carbon Co.

of the system has not been determined. Use of two traps, each with two 500-cc. flasks containing 50 gm. fresh bean material each, gave a dry product in 18 hours, with a total consumption for freezing and drying of 10 lb. of dry ice. Such samples are adequate for complete microchemical analysis. The dried material is readily ground in a Wiley Micro mill. The powder is ground to pass a 60 mesh and then stored in an evacuated desiccator over phosphoric pentoxide.

In case it is not essential to save the distillate, the apparatus described by TAYLOR and BEARD (16) can be modified by fitting the desiccant chamber with metal piping (four outlets at least) to which 34-mm. pyrex tubes (or any smaller size), each with an interchangeable female connection, can be fastened with the rubber tape. Into the female part may be fitted the flasks of any size with male 40/50 connections.

### Experimental results

By use of material of fresh and of frozen-dried roots of red kidney bean, of fresh and of frozen-dried nodules, and of frozen-dried hypocotyl of tomato and of galls produced in this organ by inoculation with *Phytophthora tumefaciens*, a comparison has been made of the activity of the wet ether extracts of these materials. The results are given in table 1. It is evident from the table that the frozen-dried material yields more potent extracts, not only in the first but also in subsequent extractions. Incidentally the extracts of nodules are much more auxinic than are those of roots. These findings corroborate earlier reports (8, 10).

A study of the role of dry ether, absolute alcohol, and water in the extraction of auxins was made, using bean nodule and tomato gall material prepared by the freezing and drying method. For controls, equal weights of the dry powder of the nodules (0.175 gm. = 1 gm. wet weight) were extracted for 5 days with 50 cc. of ether (column 1) distilled over  $\text{FeSO}_4$  and  $\text{CaO}$  in water (table 2). The other types of solvents are indicated in columns 2-9. Only the results for the nodule material are reported numerically. The ether used in columns 2-7 was distilled and stored over sodium to render it both anhydrous and free of peroxides. The water added was distilled, and boiled to remove oxygen, as was that used in column 8. The extract in each case was evaporated to dryness in a water bath at 45° C., except that of column 8 which was evaporated in the apparatus already described. This yielded a beautiful feathery residue which promptly took up water. Each residue was taken up in 2 cc. of 1.5 per cent agar. The first dilution tested therefore represents the ether extract from 0.5 gm. of wet material. The standard *Avena* coleoptile test was used, and the indoleacetic acid values for the test days are given. Since there is no unanimity among auxologists as to the unit to be used in recording the results of this test, and since all units proposed so far are subject to serious shortcomings, the data are reported without conversion into units, so that they may be used by anyone, once a unit is agreed upon.

Table 2 shows that the dry solvents, ether and alcohol, do not contain any active fractions after standing over the material for 5 days or after Soxhletization

TABLE 1

COMPARISON OF AUXINIC ACTIVITY OF WET ETHER EXTRACTS OF FRESH AND OF FROZEN VACUUM-DRIED NODULES AND ROOTS OF RED KIDNEY BEAN, EXPRESSED IN AVERAGE CURVATURE PER TWELVE AVENA COLEOPTILES (DEGREES)

MATERIAL AND TREATMENT	GRAMS FRESH MATERIAL EXTRACTED REPRESENTED BY ITS PORTION OF EXTRACT IN 1 CC. OF 1.5% AGAR	TESTING DATES										
		10/18	10/23	10/25	10/30	11/1	11/6	11/20	11/27	12/4	12/11	1/31
Nodules: fresh into wet ether 10/9/40 (1 vol. in 50)	0.0078	16.6	.....	0	.....	.....	.....	.....	.....	.....	.....	.....
	0.0156	23.5	.....	0	.....	.....	.....	.....	.....	.....	.....	.....
	0.0312	32.3	.....	0	.....	.....	.....	.....	.....	.....	.....	.....
	0.0625	29.9	.....	1.7	.....	.....	.....	.....	.....	.....	.....	.....
	0.1250	27.5	.....	9.3	.....	0	.....	.....	.....	.....	.....	.....
	0.2500	.....	.....	.....	.....	0	.....	.....	.....	.....	.....	.....
	0.5000	.....	.....	.....	.....	2.2*	.....	.....	.....	.....	.....	.....
	1.0000	.....	.....	.....	.....	10.4	.....	.....	.....	.....	.....	.....
Roots: Fresh into wet ether 10/9/40 (1 vol. in 50)	1.25	0	.....	0	.....	0	.....	.....	.....	.....	.....	.....
	2.5	0	.....	0	.....	0	.....	.....	.....	.....	.....	.....
	5.0	0	.....	0	.....	0	.....	.....	.....	.....	.....	.....
	10.0	0	.....	0	.....	0	.....	.....	.....	.....	.....	.....
Nodules: Frozen and vacuum-dried 10/9/40, into wet ether 10/18/40 (1 vol. in 50)	0.00095	.....	1.0	.....	.....	.....	.....	.....	.....	.....	.....	.....
	0.0019	.....	3.4	.....	.....	.....	.....	.....	.....	.....	.....	.....
	0.0039	.....	8.4	.....	0.5	.....	.....	.....	.....	.....	.....	.....
	0.0078	.....	.....	.....	3.3	.....	0.7	.....	.....	.....	.....	.....
	0.0156	.....	21.5	.....	10.0	.....	1.9	.....	.....	.....	.....	.....
	0.0312	.....	.....	.....	18.9	.....	4.6	.....	.....	.....	.....	1.1
	0.0625	.....	23.6	.....	23.3	.....	10.5	.....	7.4	8.6	7.3	6.0
	0.1250	.....	.....	.....	28.8	.....	19.7	19.0	17.7	12.1	17.2	17.0
	0.2500	.....	18.4	.....	30.5	.....	23.6	21.2	22.4	20.7	23.3	23.9
	0.5000	.....	.....	.....	23.9	.....	20.1	21.7	23.8	25.0	32.0	29.5
Roots: Frozen and vacuum-dried 10/9/40 into wet ether 10/18/40 (1 vol. in 50)	1.25	.....	0.2	.....	.....	.....	.....	.....	.....	.....	.....	.....
	2.5	.....	3.2	.....	6.4	.....	0.3	.....	.....	.....	.....	6.7
	5.0	.....	10.9	.....	12.2	.....	4.0	.....	.....	.....	2.3	12.7
	10.0	.....	13.6	.....	15.3	.....	9.9	9.2	18.0	17.2	6.8	19.2
Indoleacetic acid:												
20γ.....		15.4	9.4	14.7	10.7	8.8	8.1	10.2	9.0	11.8	14.8	11.2
40γ.....		24.6	14.7	22.9	20.7	20.1	14.5	15.1	21.9	16.8	19.0	25.9

\* Discontinued for lack of treating time. In preceding experiment (not recorded) activity continued at this low level from seventh to eleventh extraction.

for 3-7½ hours. The same order of results, which will be published in another paper, was obtained with tomato hypocotyl and crown gall of tomato. The table also shows that when wet ether is used, or dry ether to which water has been added, the extract is active in the *Avena* test and water alone gives a highly active extract.

In another experiment dry bean nodule material was extracted for 5 days with 50 cc. of dry ether and another lot daily for 5 days with 10 cc. of dry ether per day, the residues being pooled for the test. The ether extracts of each sample were inactive. The extracted residues were divided each into two lots and used with other samples for some of the tests recorded in table 3. It was expected that the Soxhletized material would yield very active extracts and hence it was decided to test high dilutions. Because we were mistaken in this we just caught the upper range of activity of the extracts with the lowest dilutions. Identical results were

TABLE 2

AUXINIC ACTIVITY OF ETHER EXTRACTS OF FROZEN VACUUM-DRIED NODULES OF RED KIDNEY BEAN EXPRESSED IN AVERAGE CURVATURE PER TWELVE AVENA COLEOPTILES (DEGREES) (ETHER DRIED OVER SODIUM, EXCEPTING AS INDICATED)

GRAMS FRESH MATERIAL EXTRACTED REPRESENTED BY ITS PORTION OF EXTRACT IN 1 CC. OF 1.5% AGAR	WET ETHER 50 CC., FOR 5 DAYS	DRY ETHER 50 CC., FOR 5 DAYS	DRY ETHER 50 CC. + 1 CC. H <sub>2</sub> O, FOR 5 DAYS	DRY ETHER 50 CC. + 1 CC. 100% ALCOHOL, FOR 5 DAYS	DRY ETHER 50 CC. + 1 CC. 100% ALCOHOL + 1 CC. H <sub>2</sub> O, FOR 5 DAYS	SOXHLETIZED FOR 3 HOURS WITH 240 CC. DRY ETHER	SOXHLETIZED FOR 3 HOURS WITH 240 CC. DRY ETHER + 1 CC. H <sub>2</sub> O	50 CC. OF WATER, FOR 5 DAYS
0.00105.....	8.1	0	4.9	0	2.3	0	0	0.8
0.0078.....	31.5	0	25.3	0	14.8	0	9.6	12.5
0.0312.....	38.0	0	30.5	0	25.6	0	.....	25.3
0.125.....	23.0	0	24.2	0	26.9	0	.....	.....
0.50.....	6.5	0	13.7	0	14.1	0	.....	.....
Indoleacetic acid:								
20γ.....			17.4				11.0	
40γ.....			24.9				19.7	

obtained with dried material of tomato hypocotyl and tomato crown gall, except that the numerical values are different. These will be published later.

The results of table 3 show that previous extraction of dry material with dry ether does not affect its auxin content; in fact, this may facilitate subsequent extraction with water or with aqueous ether. The relatively low yields after Soxhletization with wet ether probably are due to the short time (3-7½ hours) during which wet ether was in contact with the material. That they are not due primarily to destruction of auxin by the temperature of boiling ether is evident from the table. Table 3 also shows that the dry ether extract does not contain a fraction which can be activated by addition of water. For a time this was thought to be the case and it was assumed that this meant that dry ether extracts contain an inactive precursor (such as an ester) which is rendered active by water. This was an

error. In not one of many subsequent tests was it possible to duplicate these results, although they were repeated with ether residues of the lot from which they had been obtained. It was concluded that the original extraction was not carried out with dry ether, as previously thought, and that the thimble of the Soxhlet which was washed with wet ether had not been allowed to dry sufficiently. This suggests that small amounts of water in ether suffice to give an active extract. The minimal amount necessary has not been determined, nor the optimal time for extraction. Both nodule and tomato material show that a 120-hour extraction

TABLE 3

AUXINIC ACTIVITY OF ETHER EXTRACTS OF FROZEN VACUUM-DRIED NODULES OF RED KIDNEY BEAN EXPRESSED IN AVERAGE CURVATURE PER TWELVE AVENA COLEOPTILES (DEGREES). MATERIAL PREVIOUSLY EXTRACTED WITH DRY ETHER FOR TESTS OF TABLE 2 (ETHER DRIED OVER SODIUM)

GRAMS FRESH MATERIAL EXTRACTED REPRESENTED BY ITS PORTION OF EXTRACT IN 1 CC. OF 1.5% AGAR	RE-EXTRACTED FOR 5 DAYS WITH 50 CC. DRY ETHER+1 CC. H <sub>2</sub> O	RE-EXTRACTED FOR 5 DAYS WITH 50 CC. DRY ETHER TO WHICH 1 CC. H <sub>2</sub> O WAS ADDED AFTER EX- TRACTION	RE-EXTRACTED WITH 50 CC. DRY ETHER+1 CC. H <sub>2</sub> O	
			HALF REFLUXED FOR 3½ HOURS	HALF NOT RE- FLUXED
0.00048.....		0	0	0
0.00097.....		0	0	0
0.00195.....	11.7	0	0	0
0.0039.....	22.3		3.3	2.6
0.0078.....	36.5	0	6.7	7.6
0.0156.....				
0.031.....		0		
Indoleacetic acid:				
20γ.....			11.0	
40γ.....			19.7	

with 50 cc. of wet ether gives better results than a 24-hour extraction, or than a 7½-hour extraction in a Soxhlet with 1250 cc. of ether-water passing over the material. No complete study has been made of the amount of ether best suited for maximal extraction, nor of the role of temperature.

One extraction of dry material with wet ether does not remove all auxin (table 1). The dry powder avidly takes up the water from the solvents, swells, and becomes colloidal. The number of extractions necessary for its complete removal has not been determined (table 1). This applies both to nodule and to tomato materials. The combined extracts of a dry ether extraction and of a subsequent wet one yield a highly active preparation which, however, is neither more nor less active than the wet ether extract alone (table 4); that is, there is no evidence of synergism. The same applies to extracts of tomato gall.



Evaporation of ether-water extracts, and subsequent treatment of the residue (dried in an Abderhalden vacuum dryer<sup>6</sup>) with dry ether yields an active preparation (table 4). The same result was obtained with extracts of the tomato gall.

TABLE 4

AUXINIC ACTIVITY OF ETHER EXTRACTS OF FROZEN VACUUM-DRIED NODULES OF KIDNEY BEAN EXPRESSED IN AVERAGE CURVATURE PER TWELVE AVENA COLEOPTILES (DEGREES) (ETHER DRIED OVER SODIUM)

GRAMS FRESH MATERIAL EXTRACTED REPRESENTED BY ITS PORTION OF EXTRACT IN 1 CC. OF 1.5% AGAR	EXTRACTED 3 DAYS WITH 50 CC. DRY ETHER; EXTRACT EVAPORATED TO DRYNESS AT 45° C.			EXTRACTED 1 DAY WITH 50 CC. DRY ETHER; FILTRATE EVAPORATED AND RESIDUE STORED (A); POWDER RE-EX- TRACTED 5 DAYS WITH 50 CC. DRY ETHER AND 1 CC. H <sub>2</sub> O; EXTRACT EVAPORATED, RESIDUE (B) COM- BINED WITH (A), AND TAKEN UP IN AGAR
	RESIDUE TAKEN UP IN AGAR	POWDER RE-EXTRACTED 5 DAYS WITH 50 CC. ETHER+1 CC. H <sub>2</sub> O; EXTRACT EVAPORATED, RESIDUE DRIED IN VACUUM, THEN TREATED WITH DRY ETHER		
		SOLUTION TAKEN UP IN AGAR	RESIDUE TAKEN UP IN H <sub>2</sub> O AND AGAR	
0.000195.....	0.0	0.0	0.0	0.0
0.00039.....	0.0	1.1	0.0	0.0
0.00078.....	0.0	5.0	0.0	7.2
0.00157.....	0.0	15.6	0.0	19.0
0.00315.....	0.0	24.0	0.0	25.8
0.0062.....	0.0	27.1	0.0	23.8
0.0125.....	0.0	22.3	2.0	26.4
0.025.....	0.0	.....	8.1	24.3
0.050.....	0.0	.....	15.3	.....
Indoleacetic acid:				
20γ.....	10.0		11.2	13.3
40γ.....	18.6		25.9	18.7

### Discussion

These results indicate that the method of extracting auxins from frozen vacuum-dried material is superior to extraction of fresh material. While the fresh roots yielded an inactive extract, the frozen-dried roots gave an active extract. Also the yields obtained from frozen-dried nodules are higher than from fresh nodules. The results also indicate that water plays some role in liberating the active fraction of ether extracts of auxin from plant tissues, and that this fraction is soluble in dry ether. The findings of SKOOG and THIMANN (17) indicate that this is done hydrolytically, and that proteolytic enzymes, such as chymotrypsin, may function in the process. Our results indicate that any free auxin present in living tissues either is destroyed in the process of freezing or of dehydration, or of both,

<sup>6</sup> We are indebted to Dr. T. F. GALLAGHER for use of this apparatus.

or is fixed during these processes so that none is removed by dry ether. Possibly this may be correlated with the fact that dry tissues do not grow, that maturation involves dehydration, and that storage organs such as seeds do not yield auxins until water is added. If this is the case, then liberation and fixation of auxins in the plant are under metabolic control, and auxin itself is an agent whereby the plant controls some aspects of its growth, but is not the something which exercises that control.<sup>7</sup>

It is probable also that some of the difficulties of auxologists may have been due to the method of extracting whole fresh material by placing it in wet or in dry ether. The VAN OVERBEEK method (14) discards the water-ether mixture, which settles out if enough water is present. Much auxin may be lost by this procedure. Also the placing of fresh material in ether probably does not arrest all enzyme activity. Thus different amounts of auxin may be lost in different extractions, resulting in irregular or in negative results.

It seems probable that the difficulties others (11, 15) and we (9) have experienced in supporting the early findings (9) to the effect that crown-gall tissue of tomato contains more auxin than the normal tissue is in part due to these factors. In a later publication it will be shown that this difference does exist, as it does for all gall tissues tested by us to date. The results corroborate earlier reports (8, 10) that the nodules of the red kidney bean are much more auxinic than the roots.

If it should prove generally applicable that dry plant tissues do not yield auxin to dry ether, then this solvent may be used partially to defat and decolorize (dry ether removes chlorophyll completely) samples preceding extraction for auxins with water or watery solvents. This may prove a step toward isolation, identification, and quantitative estimate of auxins in plants. The dry material, prepared by freezing and drying in vacuo, should also prove highly useful in other chemical analyses of plant tissues.

### Summary

1. A method is described for drying frozen plant material in vacuum with a minimum of chemical alteration, which is highly desirable for auxin and other chemical analyses. Material so prepared is auxinic and gives greater yields of auxins than fresh material.

2. Ether dried over sodium does not extract auxins from the material, nor does absolute alcohol. If a little water is added to such ether or to a dry ether-alcohol mixture, an active extract is obtained. Water extracts of such material alone are highly active. The material is lyophile, speedily taking up water from the solvent.

<sup>7</sup> After this paper had gone to press, a highly pertinent paper appeared: THIMANN, K. V., and SKOOG, F., The extraction of auxin from plant tissues. *Amer. Jour. Bot.* 27:951-960. 1940. They, too, find that water is necessary for auxin extraction from dry material and that drying "fixes" the "unbound" auxin. They dried the material by heat and noted a decrease in yield of auxin due to heating.

3. The auxin liberated by water from dry material is soluble in dry ether. Water is necessary for the presence of active auxin in plant tissues. Freezing or drying, or both, apparently leads to destruction of active auxin or to its fixation in a nonremovable form.

4. Pretreatment of dry material with dry ether removes ether-soluble substances and yields a partially fat-free and ether-soluble pigment-free sample for auxin extraction. This should facilitate isolation and identification of auxins.

5. These findings so far apply to all materials tested: nodules of red kidney bean, hypocotyl of tomato, and hypocotyledonary gall of tomato incited by *Phytophthora tumefaciens*.

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# HISTOLOGICAL REACTIONS OF BEAN PLANTS TO L-TRYPTOPHANE

E. J. KRAUS<sup>1</sup>

(WITH FIFTEEN FIGURES)

## Introduction

This paper is one of a series which deals with the histological reactions of plants to various growth-regulating substances. The l-tryptophane used was secured through the Eastman Kodak Company and was applied in the usual manner as a 2 per cent mixture with anhydrous lanolin. The details of procedure were the same as given earlier (3). Only one application was made. With other lots of bean plants it has been determined that a 0.5 per cent mixture is approximately as effective as the 2 per cent, and that the histological responses are similar. As in the case of the other growth substances so far worked with in detail, a more rapid and pronounced response occurs at a relatively high humidity than at a lower one, but in the experiments here reported the humidity was always well below saturation and no attempt was made to raise it in any way.

The strain of beans used was the same as in previous experiments. These plants were grown in benches filled with fertile potting soil in the greenhouses at the U.S. Horticultural Station at Beltsville, Maryland, during the spring of 1939. The actual period of the experiment from which the material illustrated was taken extended from May 1 to July 1. The usual care and attention as to watering and other details of growing were given. The temperature ranged from 18° to as high as 30° C. for short intervals on the brightest days. For the most part there was an abundance of sunshine. The control plants made what would commonly be referred to as excellent growth, were sturdy, and remained free from fungus, bacterial, or insect infestation. The specimens were preserved in Navashin's solution, handled according to the butyl-alcohol paraffin method and sectioned at 12  $\mu$ .

## Gross responses to treatment

For the first 72 hours after treatment there is little external change except a slight paling of color near the treated surface; then enlargement takes place very rapidly. The effects are less noticeable 1-2 mm. below the surface. By the end

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of the sixth day a tumor 8-10 mm. in diameter may be formed (fig. 1). This is smooth, glistening, and yellowish white, with a distinct and abrupt depression at the center owing to lack of proliferation of the cells of the pith. Growth continues rapidly for 2-3 weeks, the tumor frequently becoming 2 cm. in diameter by this time and somewhat more dome-shaped, though never with a high crown or taper,

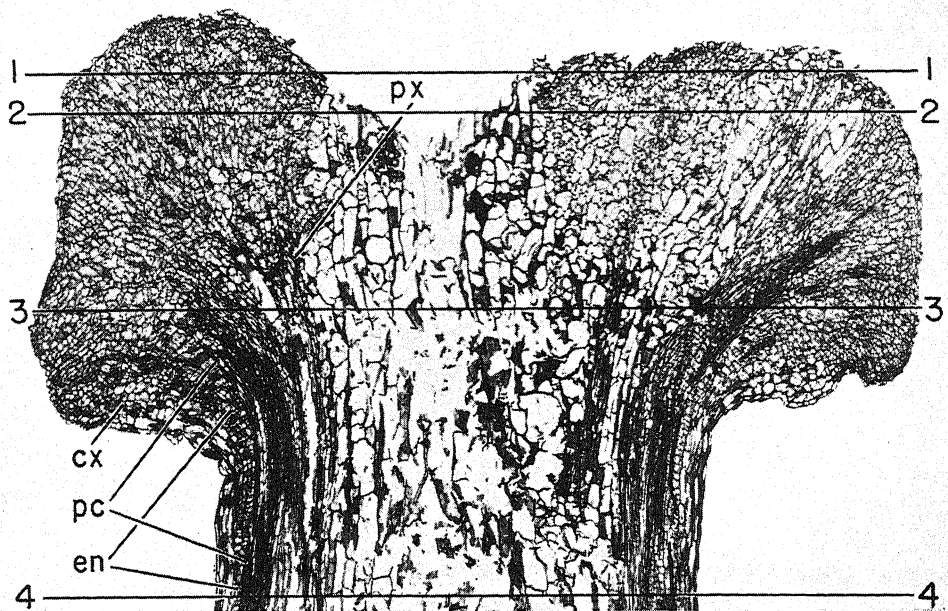


FIG. 1.—Median longisection of apical tumor 6 days after application of tryptophane-lanolin mixture: *cx*, cortical parenchyma cells only slightly divided and forced outward and downward by more actively meristematic cells centrad to them; *pc*, pericycle cells affected only slightly; *en*, greatly proliferated endodermis from whose derivatives, especially near the top, a vascular strand is being organized; *px*, primary xylem. Ends of vessels mark original surface of application. Younger parenchymatous cells of xylem, cambium, and phloem have proliferated markedly and form main portion of tumor, other than endodermal derivatives. Pith cells enlarged somewhat, then died, and became torn and disintegrated. Levels 1, 2, 3, 4 are referred to in connection with description of subsequent figures.

as in the case of those resulting from indoleacetic acid (2). Its surface is finely tuberculated, and the central depression is filled by the growth of peripheral tissues into it. The color becomes a dull gray, sometimes slightly greenish, owing to the death and suberization of some of the outermost cells and the differentiation of chloroplasts in others. After the fourth week growth proceeds slowly. The largest tumor observed at the end of 8 weeks was 2.5 cm.; most of them were appreciably smaller. Frequently large rifts occur over the upper surfaces of the tumors, owing to the death and drying out of cells. Of several hundreds of such

tumors examined, none was found with any roots or root primordia among its tissues or developing from the stem immediately below it. The tumor consists of parenchymatous cells and an intricate anastomosis of vascular strands of varying size, most of which are ultimately connected with the vascular bundles of the stem below it. A few short vascular strands are differentiated which remain completely surrounded by parenchymatous cells and are not directly connected to other vascular strands.

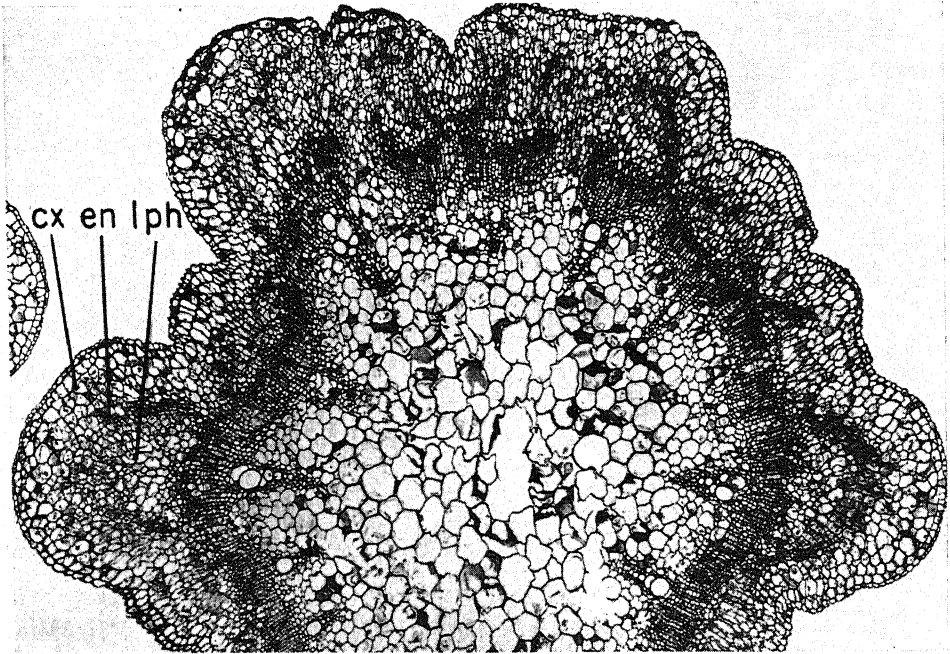


FIG. 2.—Section 1 mm. below treated surface 4 days after treatment. At left cortical parenchyma (*cx*) and endodermal cells are active, pericycle and phloem (*ph*) scarcely so, and pith has begun to disintegrate. At extreme right the endodermis proliferated, vascular bundle being organized near the rays, which are meristematic. Cambium shows varying degrees of activity.

### Histological details

The epidermal cells in close contact with the lanolin-tryptophane mixture enlarge, frequently divide radially but do not proliferate greatly, so that they are generally ruptured by the end of 96 hours by the expanding tissues within them. Close to the surface of application the cells of the cortical parenchyma proliferate somewhat abundantly within 30 hours after treatment, and in some instances their derivatives may constitute a small portion of the apical tumors developed.

The endodermis is very sensitive in its response. Within 20 hours after treatment, and near the treated surface, the cells elongate radially and tangential di-

visions may occur. Subsequently enlargement and division take place with great rapidity, so that at first—in any given plane—a broad arc of proliferating cells is found over each main vascular bundle, with the activity somewhat less over the rays. Soon this proliferating mass of cells pushes the cortical cells ahead of it and

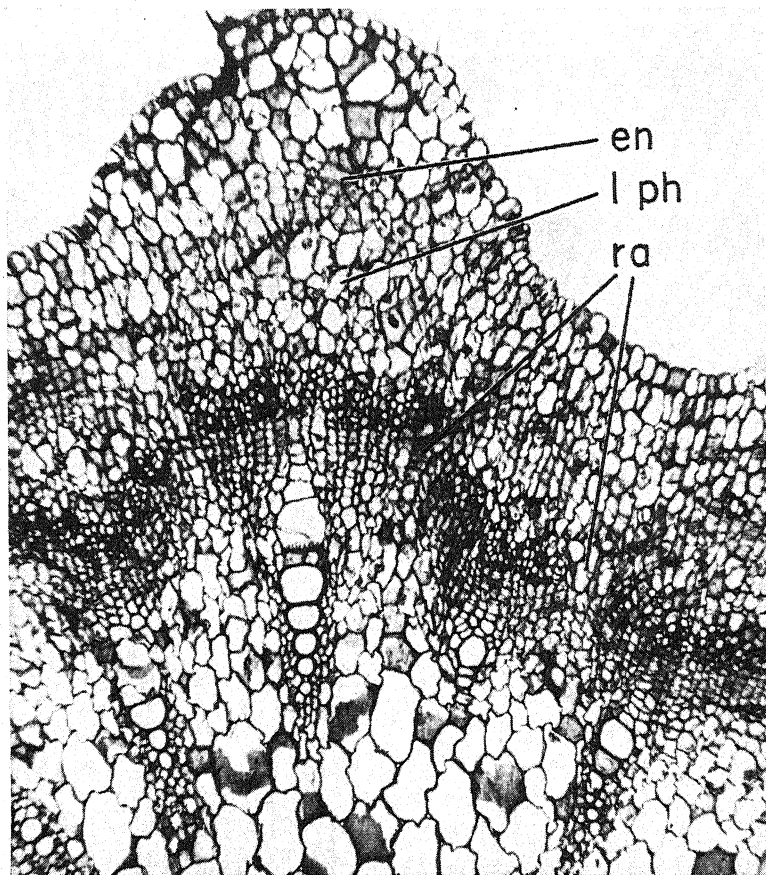


FIG. 3.—Single bundle similar to those in fig. 3, 0.5 mm. below treated surface, 3 days after application: *en*, endodermis; *ph*, phloem showing little effect; *ra*, one ray showing beginning of interfascicular cambium, other to right not yet active.

ruptures the epidermis. Throughout the mass, bands and groups of cells become differentiated as xylem and phloem tissues, with a more or less active cambium between them. These vascular strands anastomose with one another and are ultimately connected with the xylem and phloem of the stele (fig. 10). Farther down the stem, for distances of 4–5 mm. or more, the endodermis is active, progressively less so away from the treated surface. At the upper levels (figs. 2, 3) only those



endodermal cells over the apex of the primary phloem of a vascular bundle behave in a manner similar to that just described; those at the sides or margins proliferate to a less extent and give rise to vascular strands, the cambiums of which become connected with that of the stele through the progressive activity and differentiation of the cells of the medullary ray (figs. 7, 9, 10, 15).

Except immediately below the surface of application, the pericyclic cells show little response to the treatment.

The primary phloem, and to a less extent the secondary phloem, are highly reactive immediately below the treated surface, and within 72 hours after treatment (fig. 7) many derivative cells are formed. Some of these continue proliferation over a long period of time, others enlarge greatly into giant cells which may often be multinucleate, others collapse and die, and still others differentiate as vascular strands or as scattered groups of reticulate and wound tracheids (figs. 4, 11). About 0.5 mm. below the treated surface the primary phloem is active in much the same way as described, but the phloem parenchyma between the groups of sieve tube and companion cells is much less so. The type of subsequent development is similar to that at the higher levels but less extensive, although eventually a complex anastomosis of vascular strands results (figs. 12-14). At distances 2 mm. below the treated surface the cellular proliferation is less pronounced but may be appreciable by the end of 10 days (fig. 5A); at 4 mm. below there is little change in type of development, in so far as the phloem is concerned, from that of an undecapitated stem (fig. 5B).

The rays proliferate extensively at levels nearest the treated surface, and their derivatives differentiate much as do those of the phloem (figs. 4, 12, 13, 14). At slightly lower levels they are less active, and some of their derivatives form the cambium from which the vascular strands are developed between the endodermis and the vascular structures of the stele (figs. 6, 9, 15). In no case was the primordium of a root observed to arise from derivatives of the ray.

The cambium is highly sensitive near the treated surface, and after 12 hours cells derived from it divide in all planes and give rise to large masses of parenchymatous cells, some of which become extremely large and may have one to several nuclei; others continue active division, but the greater number of them mature as phloem or xylem elements, more particularly as reticulate tracheids in platelike masses and strands, and as vessels (figs. 8, 13, 14). At slightly lower levels the reaction is similar but much less marked, and at 2 mm. or more away the effects are still less obvious.

The xylem parenchyma near the treated surface is reactive in much the same manner as is the cambium. The very young vessel segments, which still possess nuclei and considerable cytoplasm, enlarge greatly, becoming enormous by comparison with the cells neighboring them (figs. 7A, 8). Frequently they divide. Some-

times they may contain several nuclei, but they usually collapse and disintegrate comparatively early in the development of the tumor. There is but little activity 1.5 mm. below the treated surface.

The pith cells respond but slightly to the treatment. Even at the treated surface those at the center of the stem do little more than enlarge for a time, then die and disintegrate (figs. 1, 4, 7). But opposite and adjacent to the points of the protoxylem of some of the vascular strands they may proliferate, continuing to do so for some days, finally wholly or in part filling in the cavity at the center of the stem resulting from disintegration of the pith and also the space above it which results from upgrowth of the cells of the peripheral tissues (figs. 1, 4, 11, 12). Only in the oldest tumors were a few traces of vascular bundles found in the tissues derived from the pith, but wound and reticulate tracheids were commonly differentiated.

Other details of the progressive development of the tumors and the several tissues which compose them are shown in the photomicrographs and the detailed descriptions which accompany them.

### Discussion

The tissue responses of the bean stem to application of tryptophane in the development of tumors in some respects are similar to, and in many others quite different from, the responses to indoleacetic acid or any of the other compounds so far recorded in detail (1, 2, 3, 4, 14). The lack of initiation of root primordia, the differentiation of large numbers of vascular bundles (particularly those derived from the endodermis), and the precise method of development of their connection with the phloem and xylem of the bundles of the stele are particularly striking features, as are also the relative lack of responsiveness of the cells of the pith, whereas many of the cortical parenchyma cells are definitely sensitive.

The application of tryptophane to several species of plants and the subsequent proliferation of tissues and formation of gall-like growths has been cited by several investigators (5, 6, 15), but no detailed accounts have been found. WENT and THIMANN (19) state that tryptophane has no auxin activity but does accelerate the growth of *Avena* coleoptiles when applied to their base, that although inactive in itself it is subsequently readily converted to the active form by plant enzymes. This assumption is deemed probable by them because tryptophane may be converted to indoleacetic acid by fungi and bacteria. Whatever the value of such assumption, in so far as it may apply in relation to *Avena* coleoptiles, the histological responses of the bean stem to tryptophane are so different from those to indoleacetic acid, even in high dilution (2, 3, 16), that there seems no justification for making it in the latter instance. As yet there is available no direct evidence

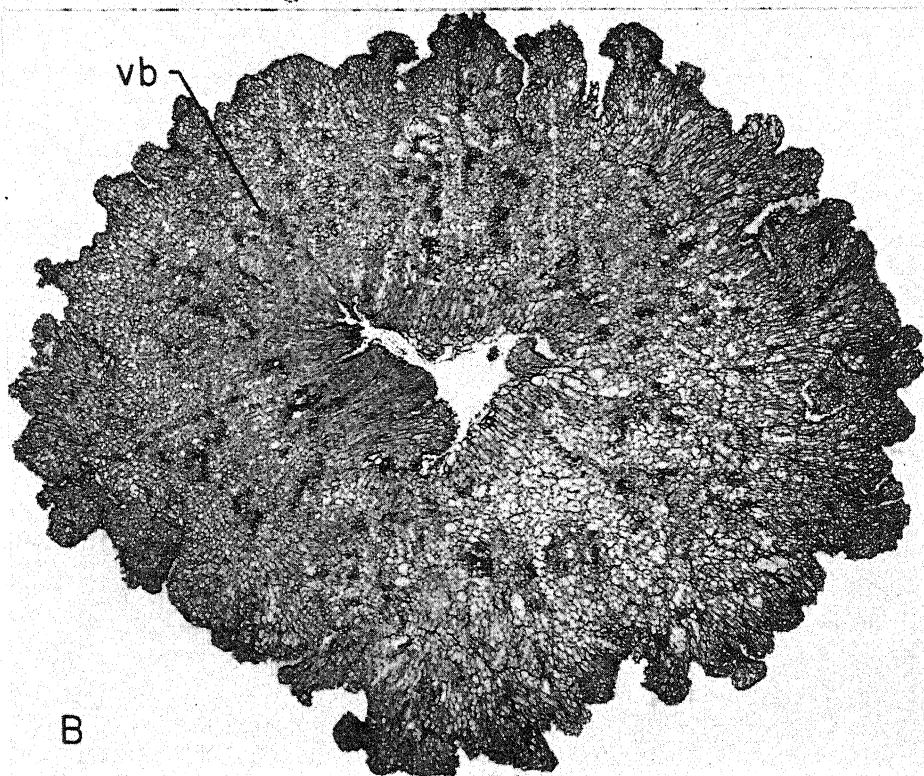
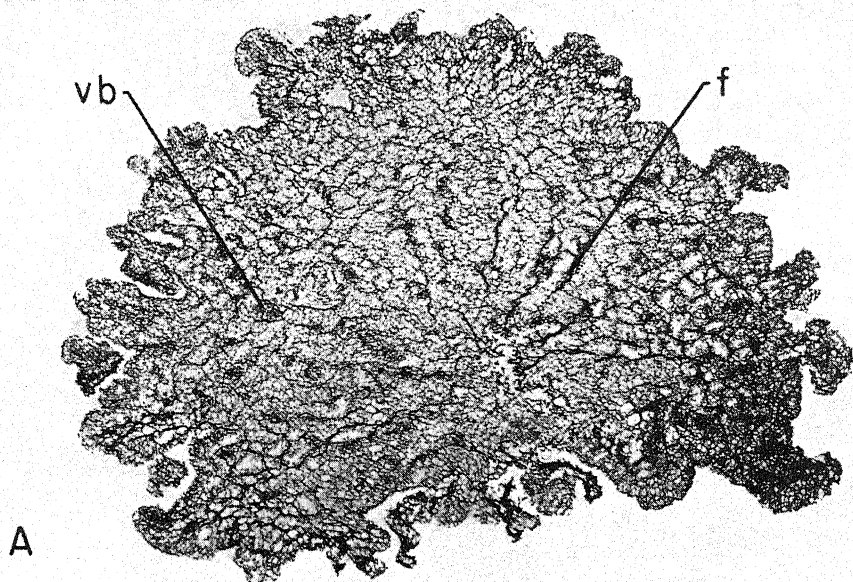
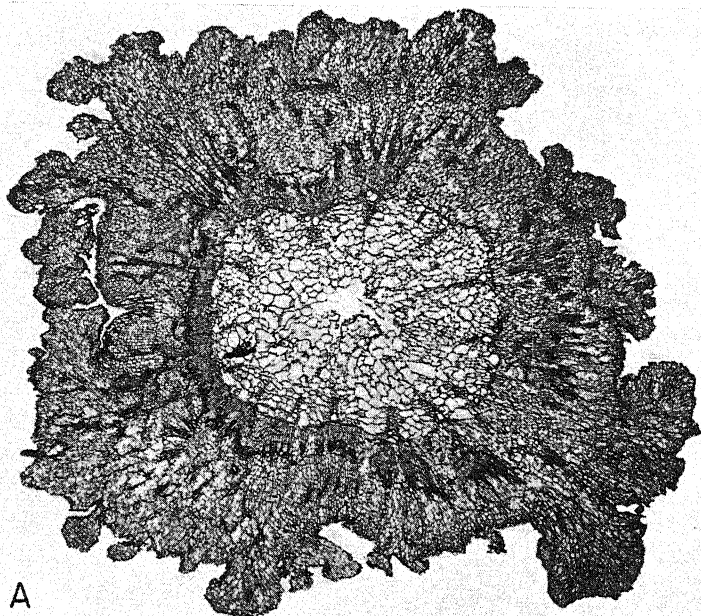
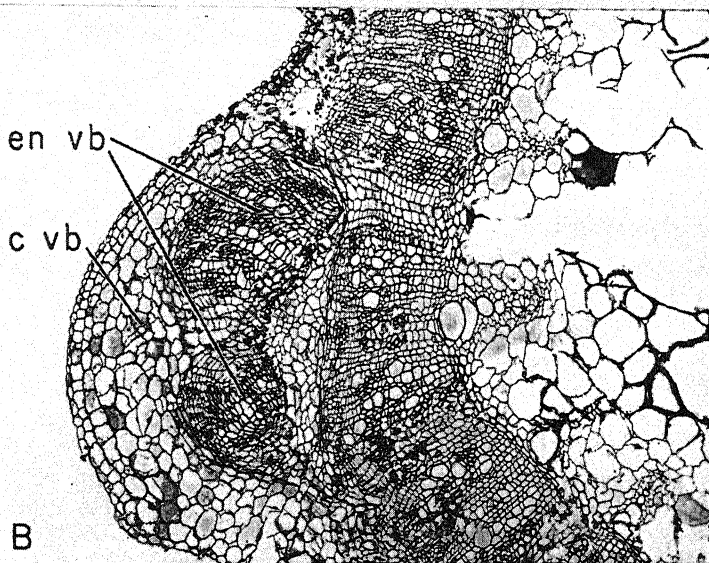


FIG. 4.—Transsections of tumor 18 days after treatment. *A*, 0.5 mm. below upper surface (approximately level 1, fig. 1): *vb*, vascular bundles differentiated from proliferated tissues; *f*, folds and strands of collapsed, dead parenchymatous cells. Between these bundles and folds are active parenchymatous cells (note particularly margins of section). No appreciable cavity at center over dead pith, this having been filled in by tissues from cells derived from xylem, cambium, and phloem. *B*, same, 0.5 mm. below



A



B

FIG. 5.—*A*, transection originally near level 3 in fig. 1, slightly more than 2.5 mm. below fig. 4*B* and below treated surface. At center is disintegrating pith, some of whose cells (opposite primary xylem) are proliferating and pushing into central cavity. Xylem already differentiated at time of application is evident. Cambium was markedly stimulated and its derivatives have matured into parenchymatous cells, tracheids of several types, tracheal segments, and at places complete vascular strands, each with separate cambium. Phloem parenchyma proliferated, and from its derivatives and those of endodermis, strands of sieve tubes and companion cells, a complex system of anastomosing vascular strands, and parenchymatous cells have been differentiated. *B*, same tumor, slightly below level 4, fig. 1. Pith disintegrated; xylem, cambium, phloem, and pericycle have developed as if untreated; from endodermal derivatives two large vascular bundles have differentiated, a small one forming from derivatives of cortical parenchyma. Somewhat below this level the endodermal bundles are directly connected with stelar bundles through vascular strands differentiated from derivatives of rays (cf. fig. 9*B*).

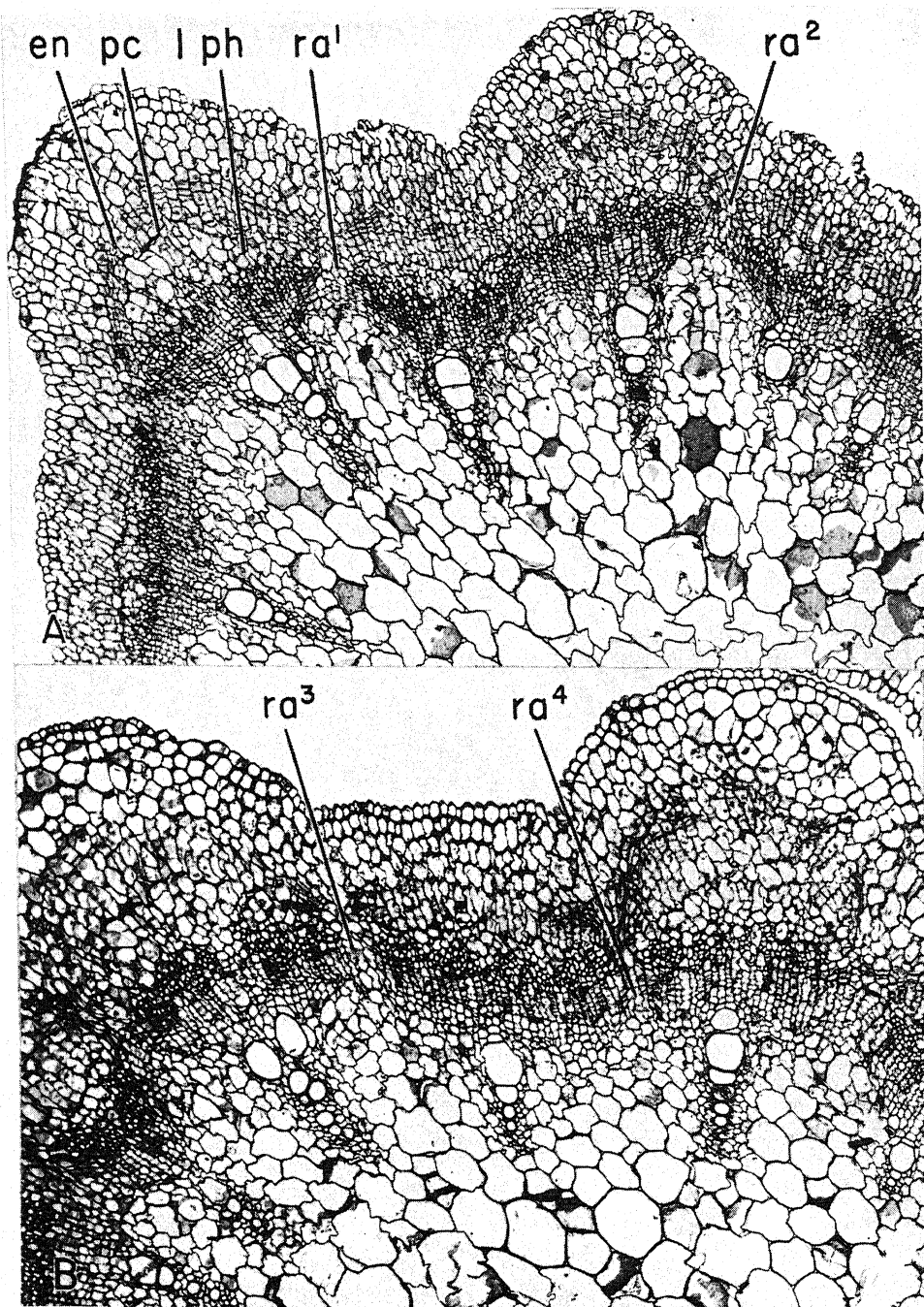


FIG. 6.—*A*, transection of 3-day-old tumor, 0.5 mm. below treated surface: *en*, active endodermis; *pc*, crushed pericycle; *ph*, slightly active primary phloem parenchyma; *ra*<sup>1</sup>, activity beginning in ray above interfascicular cambium (cf. fig. 15*B*). *B*, similar to *A*, 4 days old. *ra*<sub>3</sub>, *ra*<sub>4</sub>, without and with interfascicular cambium.

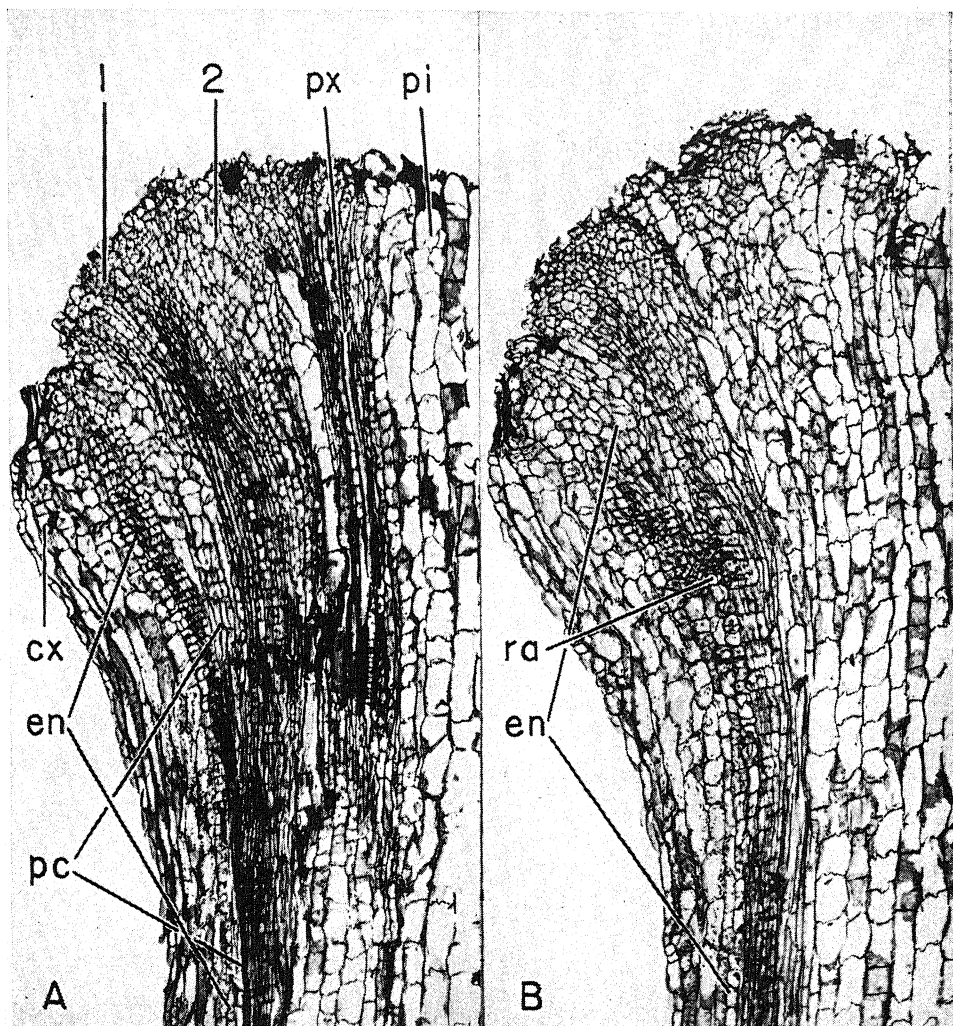


FIG. 7.—Longisection of tumor 4 days old. *A*, through midportion of bundle: *cx*, cortical parenchyma; *en*, endodermis; *pc*, pericycle; *px*, primary xylem; *pi*, pith. Except at extreme left, proliferating tissues have been considerably elevated above surface of application, indicated by dead and collapsed epidermis just above active cortical cells: *r*, cells derived mainly from phloem and phloem side of cambium; *2*, from xylem and xylem side. Large cells just left of primary xylem potential tracheal segments of secondary xylem. *B*, section through ray: *en*, endodermis; *ra*, proliferating ray cells.



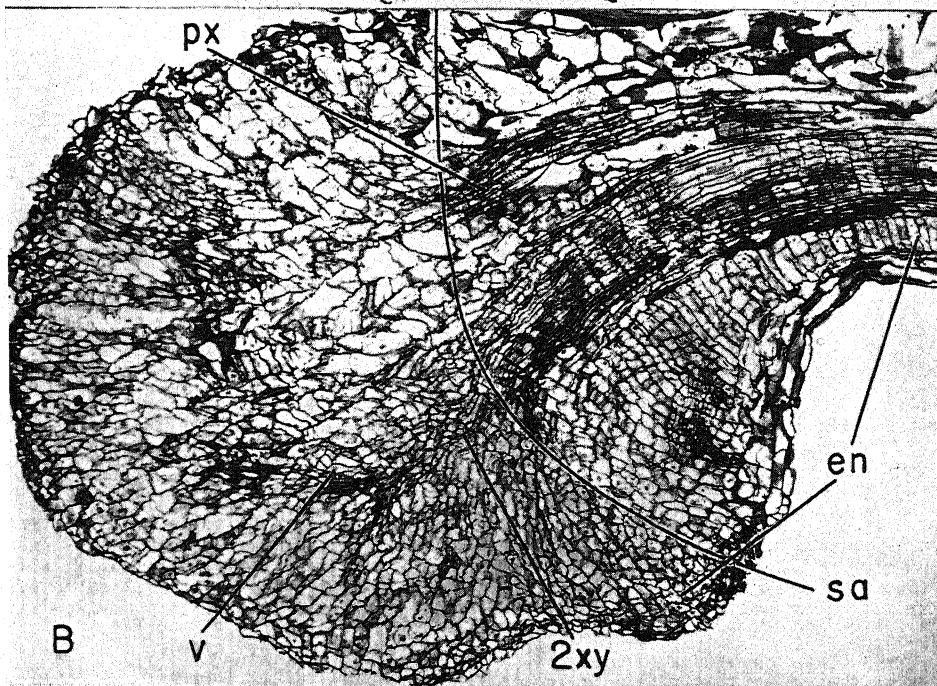
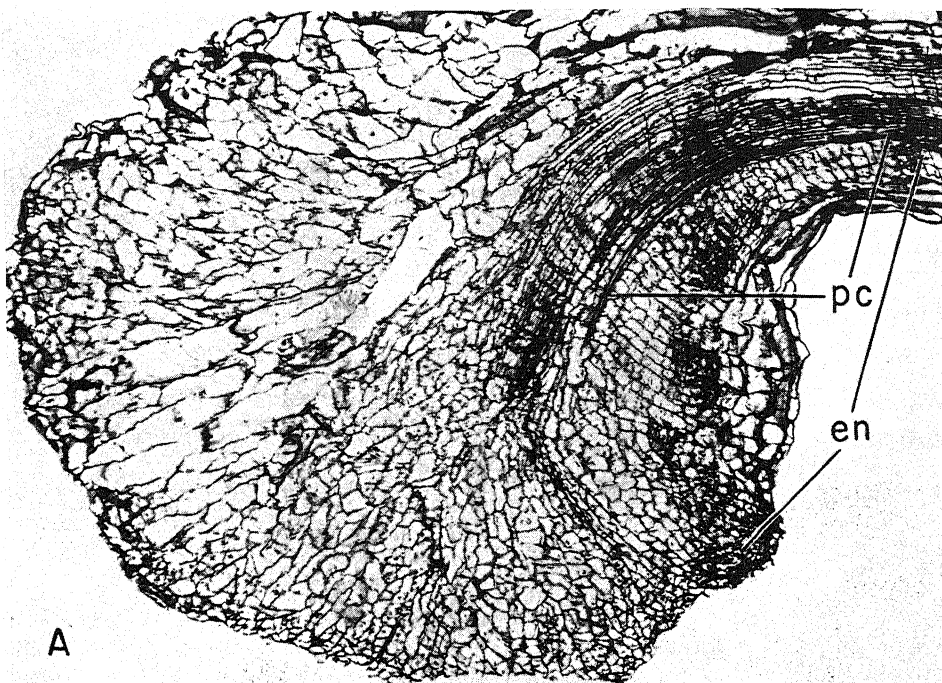


FIG. 8.—*A*, longisection of tumor 7 days old: *en*, endodermis; *pc*, pericycle. Portions of tumor derived from principal regions of stem readily distinguishable. Giant cells more conspicuous than in fig. 7. *B*, same tumor: *px*, primary xylem; *2xy*, secondary xylem showing tracheae and tracheids; *v*, vascular bundle differentiating from derivatives of proliferated xylem, others from cambium and phloem regions at lower center; large lobe of tissue derived mainly from endodermis; few derivatives of cortical parenchyma. Surface of application (*sa*) now appears curved because of differential growth of tissues.

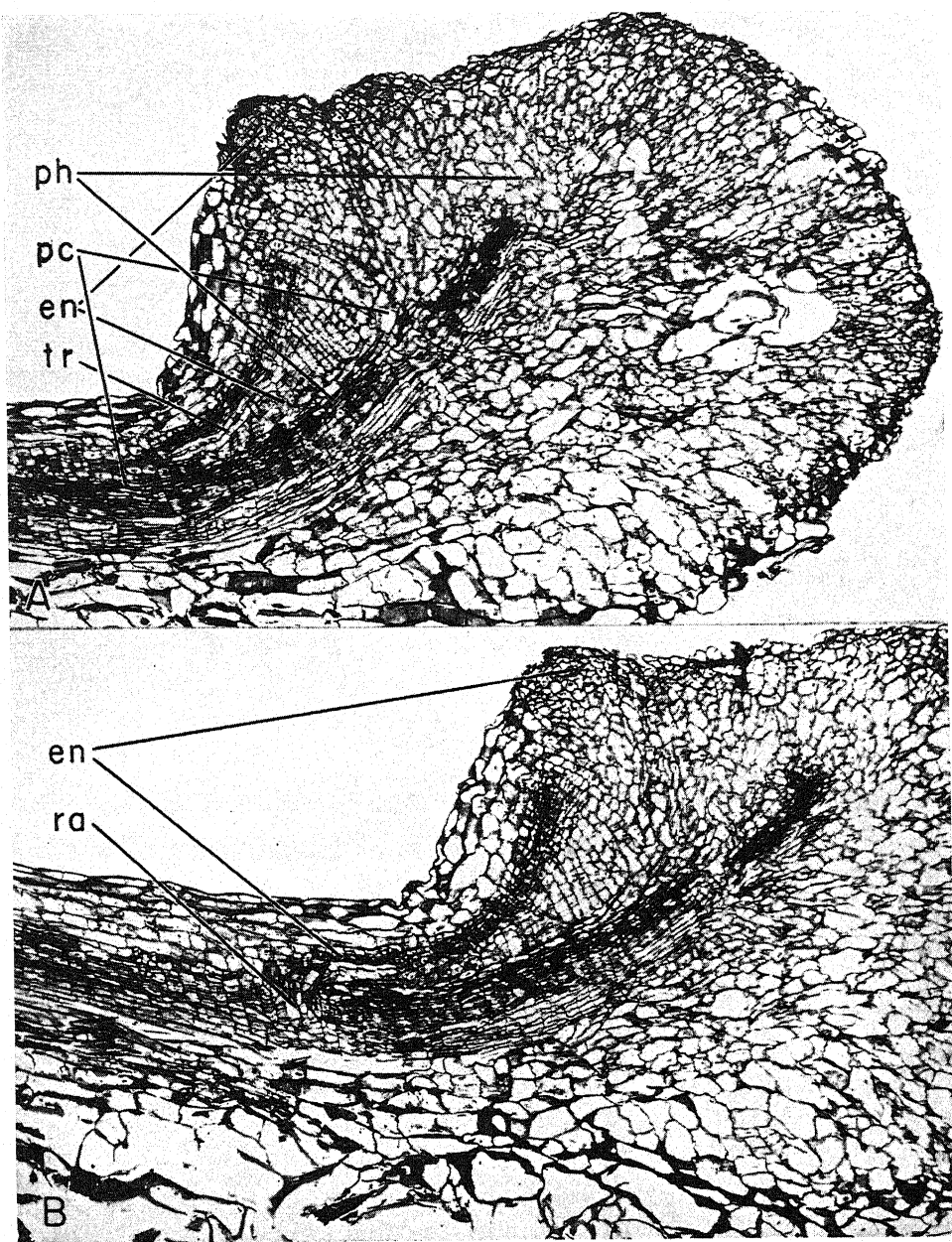


FIG. 9.—Same tumor as in fig. 8. *A*, vascular strand differentiated from endodermal derivatives: *tr*, tracheal segments and reticulate tracheids. *B*, connection of elements of vascular strand with those of stele through derivatives of ray (*ra*).



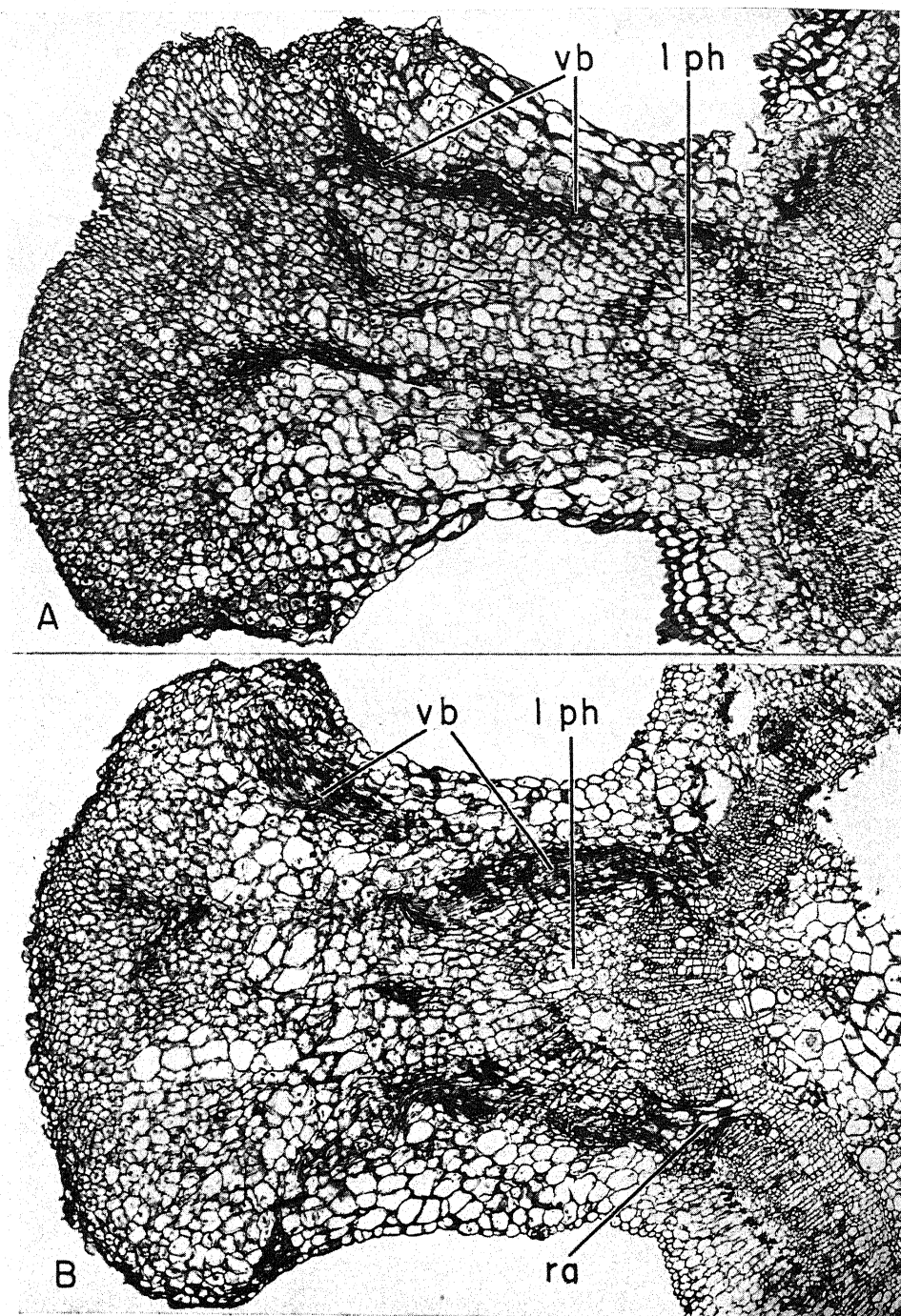


FIG. 10.—Sections through lateral projections of 7-day-old tumor near its lower margins. *A*, 1.75 mm. below upper surface. Most tissue involved derived from endodermis. Primary phloem (*ph*) slightly meristematic; pericyclic fibers pushed out of usual position but matured; other tissues centrad to these have matured as if untreated, except rays and their derivatives, from which have differentiated vascular elements connecting vascular strands (*vb*) of outgrowth with those of stele. *B*, similar section 2.5 mm. below upper surface. Most of this outgrowth is from endodermis and a few cells of cortical parenchyma which have pushed aside other cortical cells and ruptured epidermis (cf. fig. 5*B*).

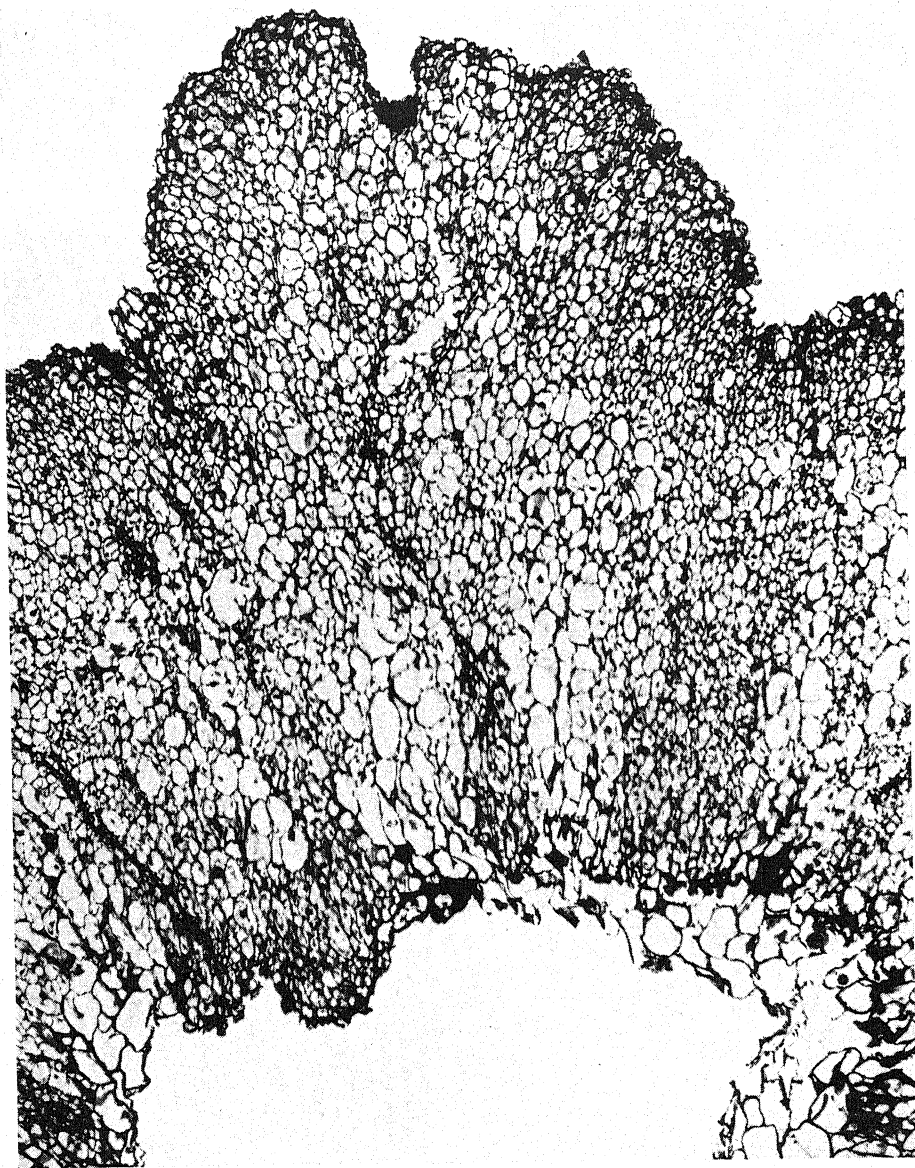


FIG. 11.—Transection of 6-day-old tumor 1.5 mm. below upper surface which has been elevated above level of surface of application (cf. fig. 8). Tissues derived mainly from phloem, cambium, xylem, and rays. At extreme bottom is cavity above pith. Dark patches and streaks are groups of sieve tubes and companion cells, and scattered here and there are tracheids, also giant cells which were potential tracheal segments, whose walls are too thin to be distinguished in photograph from associated parenchymatous cells. Groups of cells protruding into central cavity are mainly opposite and above points of protoxylem. The tear is characteristic; at later stages large holes and folds of dead collapsed cells develop.

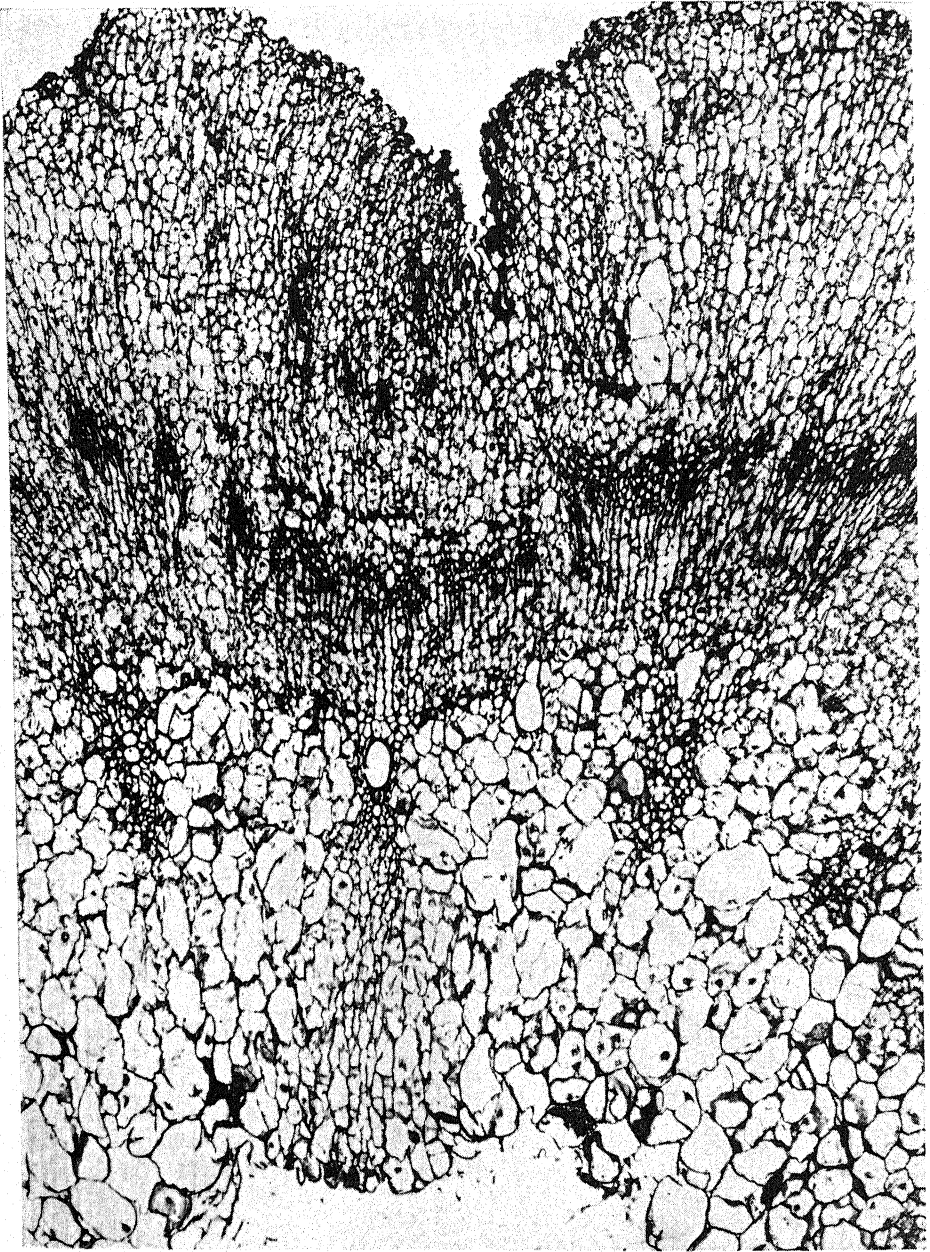


FIG. 12.—Same tumor as fig. 11, approximately 2 mm. below upper surface and just slightly below level of original surface of treatment. Pith cells are collapsed at center but somewhat meristematic opposite some points of primary xylem; cambium very active, its derivatives maturing into both phloem and xylem elements, many of which appear in semilongitudinal view because of upward thrust of tissues. Ray cells greatly enlarged and divided to some extent. Primary phloem parenchyma and endodermis have divided rapidly; some giant cells which generally collapse and die; other derivatives mature as vascular elements; still others continue rapid division and give rise to additional vascular strands and parenchyma. There are scattered tracheids, most of them reticulate; true vessels are numerous in vascular strands differentiated from proliferated tissues.



FIG. 13.—Transection of tumor 11 days old, about 2.5 mm. below upper surface and below original treated surface. Details similar to fig. 12. Tissues more clearly defined.





FIG. 14.—Same tumor as fig. 13 at slightly higher level, showing result of greater degree of proliferation of cambium and phloem with subsequent differentiation of anastomosing vascular strands and their relation to xylem of stele. Just below this level the appearance would be more nearly as in fig. 13, indicating connections of phloem. There is even greater variation in intricacy and pattern of differentiation at higher levels, but almost entire vascular network is directly or indirectly connected to vascular structure of stele.

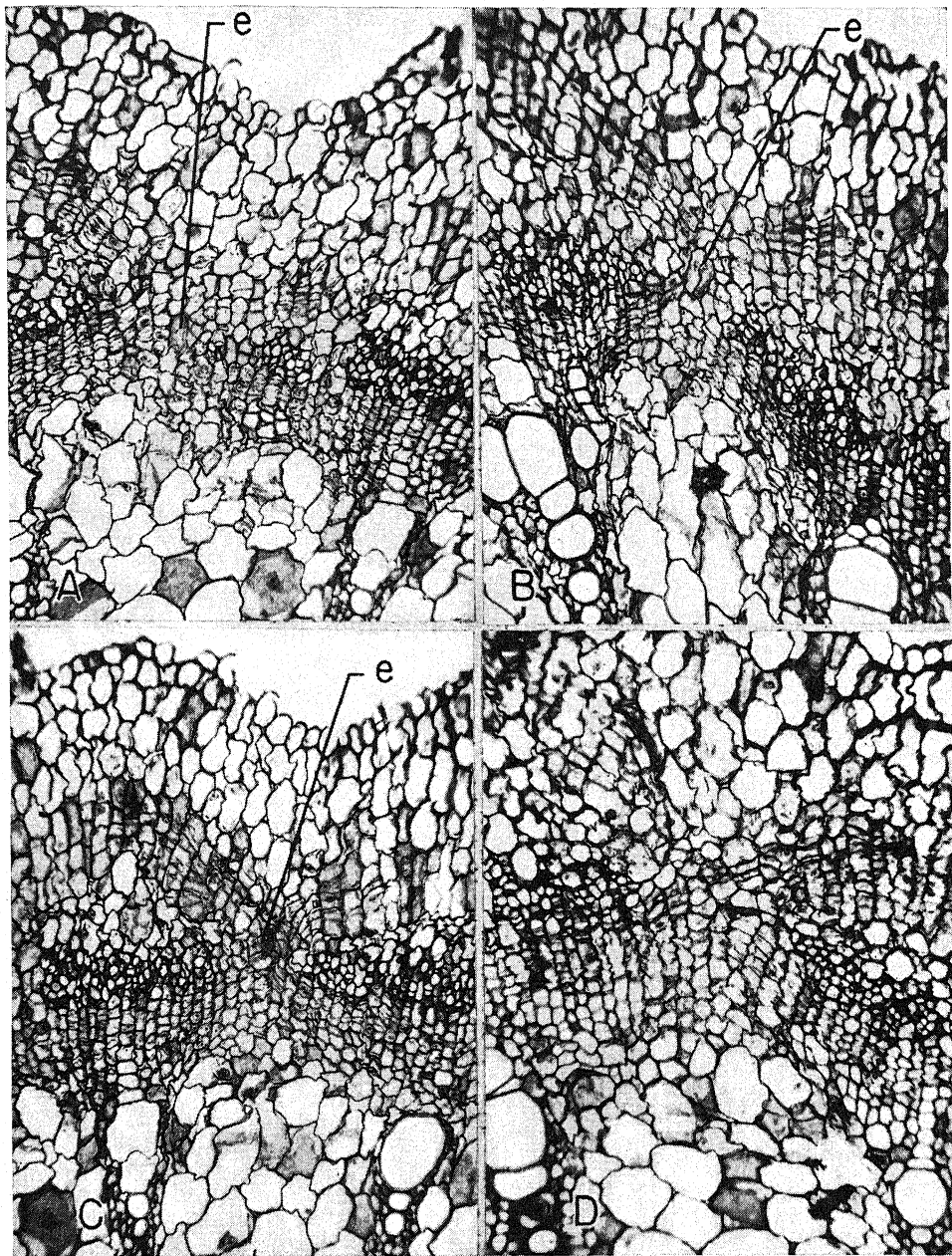


FIG. 15.—Details of development of connection of proliferating cells of endodermis with cambium of stele through activity and differentiation of ray cells. *A*, embryonic ray cell; *B*, first division of same; *C*, ray cells which have become meristematic on a line with proliferating endodermal cells over two adjacent bundles and just above similar cells in which interfascicular cambium is arising; *D*, entire group of embryonic cells forming continuous cambiums.

as to the actual substance or substances present within the cell which are responsible for the cell responses and tissue patterns which are differentiated in the case of indoleacetic acid, tryptophane, or various others, when supplied externally. There is evidence, however, that there are substances which—whether or not they show auxin activity when tested according to the *Avena* method (12)—do result in definite and characteristic responses in proliferation and subsequent patterns of differentiation when supplied externally to a rather wide variety of plants, either in a mixture with lanolin or in aqueous solution (4, 12, 16). This calls into question the value of the *Avena* test method as a precise or accurate means either for measuring or for determining quantitatively or qualitatively the substances responsible for the development of tumors caused by *Bacterium tumefaciens* (8, 9, 15), root nodules associated with rhizobia (7, 17, 18), or similar structures—provided auxin activity as thus measured is assumed to be primarily an indicator of the causes of the responses shown. It may well be that some of the substances involved may be measured in part, but it is just as likely that there are other effective compounds which escape measurement. Of the possible number of compounds which may be involved, some may show little or no auxin activity according to the *Avena* test. The complexities involved and already on record need no repetition here. Whether indoleacetic acid is or is not primarily responsible for the cellular responses observable in crown gall can no more be determined by such a test method (15) in itself than solely by the observance of the developmental sequences following treatment of plant parts with a 3 per cent lanolin-indoleacetic acid mixture or a 0.0001 per cent solution of it in water (16). The results following treatment with tryptophane as here presented indicate that it might well play a part. Its occurrence is general in living cells, and its concentration within any given cell or tissue might well fluctuate widely under various circumstances. The effect of growth-regulating substances within the plant, or outside it but in contact with it, may exert definite effects on the mobilization or accumulation of tryptophane, as has been indicated for more generalized groups of compounds (11, 12, 13, 16). The effects of varied conditions of environment, or planes of metabolism, and of the activity of organisms within or adjacent to the living tissues, are likewise important factors. To what extent tryptophane might become concentrated in tissues, and what would be the effects on development of such varied concentrations, are speculations which may well wait upon further experimental evidence, but consideration of the matter should not be omitted in relation to problems concerning growth and differentiation.

Critical data to show to what extent indoleacetic acid may be responsible for the development of the tubercles on the roots of leguminous plants in association with rhizobia are meager. Some of the suggestions are based upon the measure-

ment of auxin activity according to the *Avena* test, others on the occurrence of excrescences on roots of leguminous plants following treatment with indoleacetic acid. Neither method furnishes either effective or sufficient evidence (7, 8, 17, 18). The course of development of tubercles on roots of leguminous plants has been partially described by several investigators (10, 20, 21). Through the courtesy of Dr. LOUISE WIPF I have had opportunity to observe a series of preparations which, while incomplete, indicate clearly—as do the results of the investigations above—that the tubercle or nodule is not a potential lateral root whose development has been arrested. In the slides examined the early cellular proliferations are in the cortex, and the subsequent differentiation of the vascular bundle—which is closely associated with the mass of cells containing the rhizobia and others which are actively proliferating—is very similar to the pattern followed in the differentiation of vascular bundles from the proliferated cells in the bean stem after treatment with the lanolin-tryptophane mixture. Especially striking is the similarity between the sequence of steps resulting in the ultimate connecting up of the bundles through the pericycle to the xylem and phloem of the stele of the root to the sequence followed in the stem when a comparable connection is made through the activity and differentiation of the cells of the ray (fig. 15). This is not to say that tryptophane may be the primary causative agent or substance in tubercle formation and differentiation, any more than indoleacetic acid may or may not be. It is possible that both are concerned, and other substances as well. But it is clear that the usual tests for auxin activity are far from adequate in arriving at a decision. So far as I am aware there is no recorded detailed account of histological studies on the reactions of the roots of leguminous plants to growth-regulating substances. A short time spent on the preparation, study, and interpretation of suitable material could and would definitely establish the course of development and the histological pattern of the nodule when rhizobia are present. Similar studies in relation to growth-regulating substances would furnish suggestions as to their possible direct relation to nodule formation or their effects upon the mobilization of those substances which are directly involved.

It is not essential to adduce other examples here, but it is evident that the explanations relating to the problems of tissue development and differentiation should not be oversimplified, nor based on results from the employment of any single technique. Generalizations must await additional detailed evidence from the chemical, physiological, developmental, and histological points of view. What significance these various results may eventually have in studies relating to cancer can be indicated better when many more of them are available.



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# ANTHEROZOIDS OF SOME OF THE CHARACEAE

W. N. STEIL

(WITH SIX FIGURES)

## Introduction

The antherozoids of the Characeae have been studied by a number of workers, including BELAJEFF (1), MOTTIER (3), MEVES (2), MÜHLDOF (4), and SASAKI (5), and there is considerable difference of opinion, not only in regard to the nature of the body but also in regard to the attachment of the cilia. It was the object of this investigation to obtain more knowledge of the antherozoids of some of the Characeae.

## Material and methods

A number of species of *Chara*<sup>1</sup> and a single species of *Nitella*<sup>1</sup> were collected in the vicinity of Milwaukee, Wisconsin, between the middle of June and the middle of September, and brought into the laboratory for the study of both the living and the fixed and stained antherozoids.

Portions of the main axis of a plant bearing dwarf branches with the reddish colored antheridia were removed with small scissors and washed in tap water. A branch bearing the sex organs was next removed from the main axis and placed into a small amount of water on a slide. With the aid of dissecting needles, an antheridium was detached and transferred to a drop of water on another slide, and opened with the needles so that the antheridial filaments were exposed. This slide was placed into a petri dish, the inner surface of the cover of which had been moistened with water. The slide was removed at intervals during a period of about 40 minutes and examined under the low power of the microscope.

It was usually possible to determine the stage of maturity of the antherozoid, which when ripe is composed of distinct coils. When the antherozoids were observed to be actively motile, they were studied in the free swimming condition, or they were fixed and stained. The study of the actively motile antherozoids was facilitated by the fact that all the antheridia of the branches at a node usually reach maturity at the same time.

It was possible to make a more detailed study of the antherozoids in prepared slides. They were fixed in the usual way by inverting a slide with a drop of water containing motile antherozoids for about 2 minutes over the fumes of a 2 per cent osmic-acid solution. The slide was then set aside to dry.

<sup>1</sup> One of the species is undoubtedly *foetida*; the other species of *Chara* and the species of *Nitella* remain unidentified.

The technique for staining the antherozoids was similar to that already described (8). The schedule was as follows:

1. Place slide into a saturated tannic-acid solution in a Coplin for about 1 hour. This step may be omitted, but the solution facilitates staining the cilia.
2. Rinse in water.
3. Stain with safranin for 2-4 hours.
4. Rinse in water.
5. Dip slide into an acidulated alcoholic solution prepared by adding one drop of concentrated hydrochloric acid to 100 cc. of 95 per cent ethyl alcohol.
6. Rinse in water.
7. Stain with fast green.
8. Dehydrate at once with absolute ethyl alcohol.
9. Clear in clove oil.
10. Complete the slide in the usual way.

No observable alteration in the nature of the body or in the thickness of the cilia of the antherozoid was produced by the tannic acid. The safranin was obtained from the Coleman and Bell Co., certification number CS-6. One gm. of the dry stain was dissolved in 100 cc. of 50 per cent ethyl alcohol. The fast green was obtained from the National Analine and Chemical Co., certification number NGF-2. One-half gm. of the dry stain was dissolved in 100 cc. of 95 per cent ethyl alcohol.

The use of the acid solution is important since it not only destains the slime discharged from the antheridium and the cytoplasmic portions of the antherozoid, but it intensifies the stain in other parts of the body. A number of other combinations of stains were tried but none were found so satisfactory as the safranin-fast green combination. The nucleus, the "plastids" in the vesicle, and a small nearly spherical body at the extreme anterior end, all stain a brilliant red; other portions of the body and the cilia stain green.

### Observations

The antherozoids were occasionally discharged immediately after the dwarf branch bearing antheridia was placed on the slide, but usually a few minutes elapsed before they emerged, the time interval depending upon their maturity. In most instances motile antherozoids were obtained within 20 minutes; they were never obtained later than 1 hour.

As is well known, a portion of the wall of each of the mother cells of the antheridial filament becomes gelatinized, forming a pore through which the antherozoid is set free. The change in the wall occurs when the antheridium has opened and the gelatinous substance within it and completely surrounding the filaments has

come into contact with the water. Although the origin of this substance has not been determined, it is probably the result of dissolution of the walls of the antherozoid mother cell. A study of the form and motions of the antherozoids is not difficult, on account of their large size and because the slime at first retards their movements.

An antherozoid, at first tightly coiled, assumes when set free a slow clockwise (figs. 1, 2) or counter-clockwise rotary motion (figs. 3-5). The number of coils varies from about three to four. The rotary motion becomes rapid only after it emerges from the slime. The rapidity of the movement soon decreases as the antherozoid becomes more loosely coiled (fig. 3), and ceases when it becomes completely uncoiled. The period of time of its free swimming motion is short, usually only 3-5 minutes. Since antherozoids which are discharged naturally also exhibit free swimming motions for similar short periods of time, the technique employed here evidently did not affect the duration of their swimming movements.

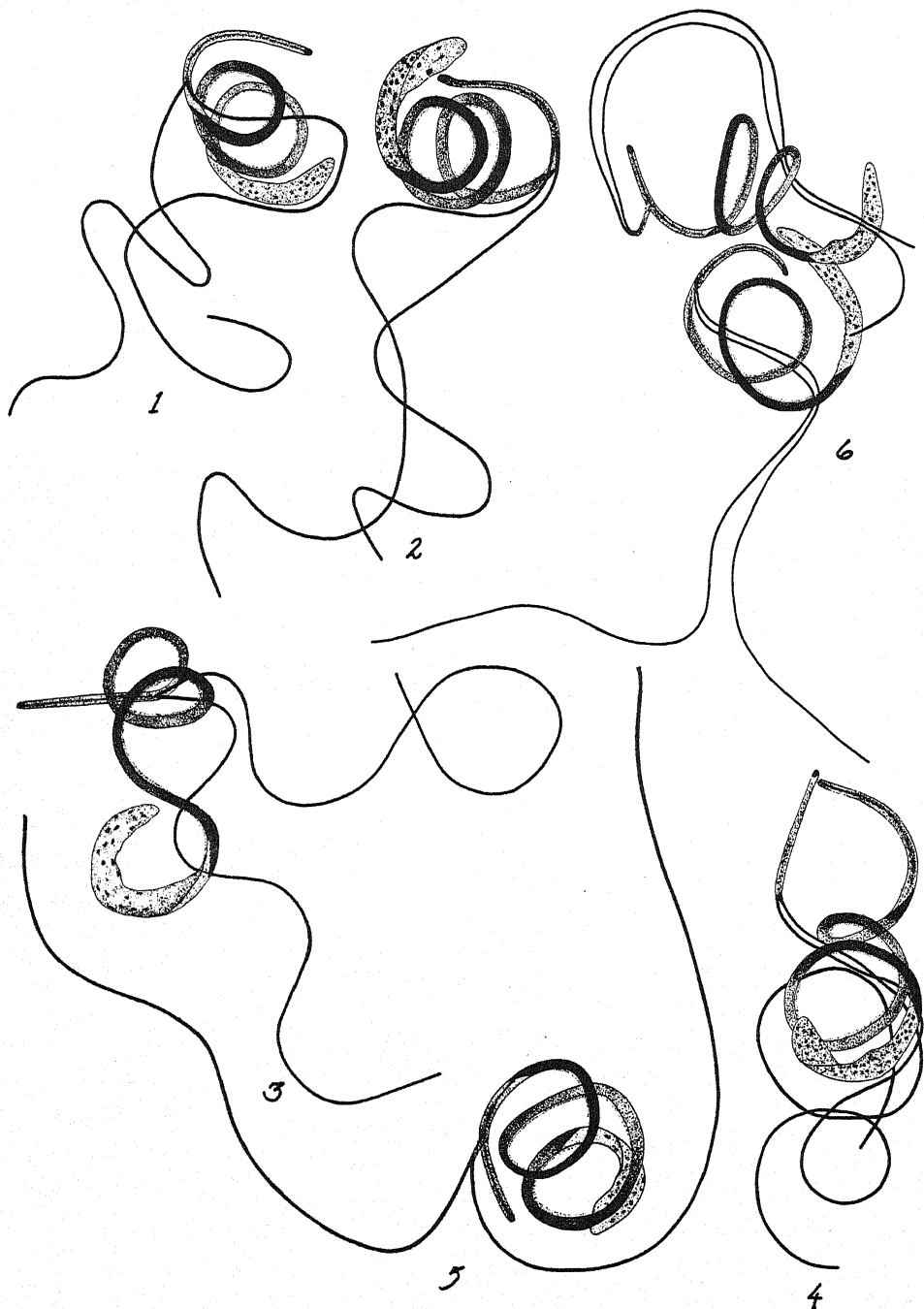
The movements of the cilia can readily be followed in the living antherozoid, although it is not possible to observe the differences in their lengths. That the cilia are attached for a considerable distance back of the anterior end, however, can be observed during the whole period of active motion of the antherozoid.

The body of the antherozoid is somewhat bandlike. The elongated nucleus (figs. 1, 2) extends from a point a short distance posterior to the place of attachment of the cilia to the large vesicle. The anterior end of the nucleus is considerably smaller in diameter than the posterior end. Both ends taper to a sharp point. A considerable portion of the anterior end of the antherozoid is cytoplasmic, as is shown in all of the figures.

The large vesicle (figs. 1-6) at the posterior end can readily be observed, even in the living antherozoid. After the antherozoid has been active for a few minutes it becomes somewhat spherical, then collapses, and is finally lost. The vesicle contains numerous red staining bodies which have been described by some workers as plastids. These "plastids" in the vesicles of different antherozoids vary in form, size, and number. In the vesicle of the antherozoid shown by figure 2 they are not only numerous but also large. To retain the vesicle it is necessary to fix the antherozoid a short time after it has been set free. A cytoplasmic portion (figs. 1-6) which extends the length of the antherozoid and completely surrounds the nucleus can be seen in the stained antherozoid.

The foregoing observations in regard to the nature of the body of the antherozoid, confirmed by the writer, have been made by a number of investigators.

The presence of a small, nearly spherical body (figs. 1-6) at the anterior end, not heretofore described in the Characeae, can be observed in the slides which have been prepared according to the technique described. Its origin and function have not been studied.



FIGS. 1-6.\*—Figs. 1-5, *Chara*: Fig. 1, actively swimming antherozoid showing nucleus, cytoplasmic portion containing small spherical body, and lightly staining thread; the two broad cilia are of unequal length; the vesicle at posterior end containing numerous "plastids" and the plasmic portion on "inner" side of antherozoid are also shown. Fig. 2, antherozoid in free swimming condition similar to that of fig. 1; "plastids" in vesicle much larger than those in fig. 1; cilium at left is the longer. Fig. 3, outstretched antherozoid which has ceased its forward rotary motion. Fig. 4, antherozoid from which ciliary band has been removed; small body at anterior end shown in the band. Fig. 5, antherozoid showing ciliary band partially detached. Fig. 6, *Nitella*: ciliary band in one antherozoid partially removed. X1750.

The cilia, as has been described by most investigators, are attached at the same point a short distance anterior to the forward end of the nucleus. As already reported (9), they are of unequal length. They appear to be attached to a band—or are united to form a band—which extends to the anterior end of the body of the antherozoid and which is in close contact with this other cytoplasmic portion. The band, when detached, is about the same width as the anterior portion of the body. The cilia are unusually thick and together equal the band in width. Occasionally in the preparation of the slides the band was partially or completely detached from the anterior end of the body (figs. 4-6). When a needle was moved about in the drop of water containing motile antherozoids, often the band became partially or wholly detached from the body (fig. 4). The thick, elongated cilia probably adhered to the needle and hence the band was readily separated. The red staining body, already described, always remained in this detached band (fig. 4).

The cilia-bearing band, which has been the principal subject of this study, was described by MÜHLDOFF (4) as an extension of the anterior end of the body. According to his description the antherozoid, at first closely coiled when set free from the mother cell, becomes outstretched, and the portion of the band in close contact with the anterior end unfolds and extends forward before the antherozoid begins its active swimming movements. According to MÜHLDOFF, therefore, the cilia are attached at the anterior end and not some distance from it. SASAKI (5) is in agreement with MÜHLDOFF. As the result of careful study, however, the writer could not confirm these observations.

A faintly staining thread (figs. 1-6) was frequently observed in the anterior end of the body. It could be more readily distinguished in those antherozoids which were slightly destained in the acid-alcohol solution. No definite statement can be made concerning the nature and origin of this structure and the small body at the anterior end until a study of spermatogenesis is made.

The antherozoids of the single species of *Nitella* (fig. 6) studied, although somewhat smaller, closely resemble those of the *Chara* species. The figure represents antherozoids having a clockwise rotary motion.

The antherozoid of the Characeae is similar to that of *Riccardia*, in which a difference in length of the two cilia was first reported by the writer (7), and to that of some other Jungermanniales reported by SHOWALTER (6) and MÜHLDOFF (4).

### Summary

1. The antherozoid of the Characeae studied is composed of about three to four coils. Its body is a somewhat flattened band which consists of an anterior cytoplasmic portion, a posterior vesicle, a cytoplasmic envelope, and an elongated nucleus which tapers to a sharp point at each end.

2. The anterior cytoplasmic portion consists of an outer and an inner bandlike part. The outer band bears the two cilia of unequal length and a small nearly spherical body at its forward end. In the inner portion a delicate thread can be seen.

3. The swimming motion of the antherozoids of *Chara* and *Nitella* is rotary, either clockwise or counter-clockwise.

4. The antherozoids of the different species of *Chara* studied are similar in every respect. The antherozoid of the *Nitella* species resembles that of *Chara*, but is considerably smaller.

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## MECHANISM OF MITOSIS IN POLLEN TUBES

KARL SAX AND J. G. O'MARA

(WITH TEN FIGURES)

The mitotic division in the generative cell of the pollen tube of *Lilium* and many other genera has long been known to be unique. Although investigated frequently since the early work of STRASBURGER and NAVASHIN, certain features of this division remain obscure in spite of an abundance of relevant data. The nature of the spindle mechanism and the formation of an equatorial plate at metaphase are problems of considerable importance in relation to the mechanism of mitosis. In the division of the generative nucleus in pollen tubes of *Lilium*, a well-defined spindle has been described by GUIGNARD (3), STRASBURGER (12), HERRIG (4), and COOPER (1), but no spindle fibers were observed by SARGENT (9), NAVASHIN (6), WELSFORD (16), O'MARA (8), and WULFF (17). According to COOPER the chromosomes always form a normal equatorial plate, but irregular orientation of the chromosomes at metaphase has been described by STRASBURGER (12), NAVASHIN (6), WELSFORD (16), HERRIG (4), O'MARA (8), and WULFF (17). The results of TRANKOWSKY (14) are especially interesting, since they show that no type description will fit all nuclear divisions in the pollen tube. In *Galanthus* and *Convallaria* the division is irregular, with no evident spindle fibers and no regular equatorial plate. In *Hemerocallis flava*, however, an equatorial plate and a typical spindle are formed. Their occurrence in this species seems to be related to the size of the elements involved. In the other cases the tube is too narrow to accommodate a division figure of normal proportions, and an atypical figure is formed. According to UPCOTT (15) the division of the generative nucleus in *Tulipa* is regular, although the equatorial plate may be oblique or buckled.

Much of the disparity in the descriptions of mitoses in pollen tubes is due to differences in the materials used, although different results have been reported for the same species. Evidently variations do occur, and it is the irregular behavior that is of particular interest in relation to the mechanics of mitosis. In dealing with the features peculiar to this division, consideration must be given to the general process of which this particular division can be only a modification.

Divisions of the generative nucleus have been examined in the pollen tubes of *Lilium regale* and other species, *Tradescantia paludosa*, *Bellevallia romana*, *Scilla* sp., *Gasteria* sp., and *Zephyranthes* sp. *Lilium* pollen tubes were fixed in the styles at various times after pollination. Those fixed in alcohol-acetic were dissected out of the style and stained with aceto-carmin. Others were fixed with Flemming's solution and other standard fixatives, sectioned, and stained with Heidenhain's



haematoxylin. Some of the *Lilium* pollen grains were grown in stigmatic fluid, and at the time of nuclear division the tubes were fixed in aceto-carmin. Pollen of the other genera were grown on slides, following NEWCOMER's (7) technique, and were fixed and stained with aceto-carmin or fixed with a modified Flemming's solution and stained with the triple stain or with Heidenhain's haematoxylin.

The analysis of the generative nucleus division in *Lilium* was done by the junior writer while he was a graduate student at Harvard University.

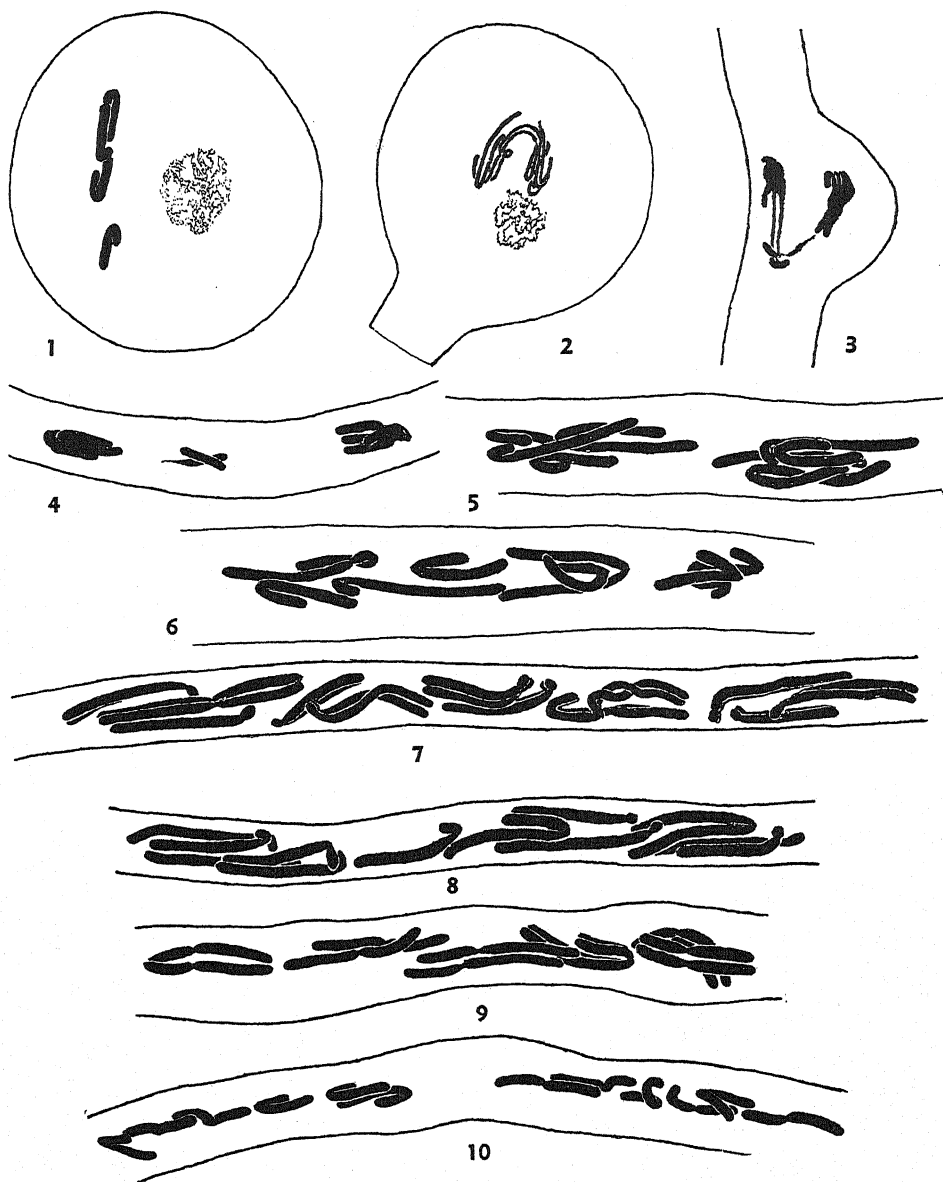
#### DIVISION OF GENERATIVE NUCLEUS

Of the various genera examined, normal division figures were characteristic in *Bellevia*, *Scilla*, and *Zephyranthes*. The centromeres were oriented on a rather definite equatorial plate at metaphase, with the chromosome arms extending lengthwise in the pollen tube. At anaphase the daughter chromosomes were uniformly distributed to the poles in a regular manner. No clearly defined spindle fibers were observed, but a definite spindle mechanism is operative.

A number of exceptional division figures were observed in *Bellevia*. The generative nucleus is compact and stains intensely in the mature pollen grain, and the tube nucleus appears to be disintegrating. Occasionally the individual chromosomes are clearly observed, indicating their orientation in the compact generative nucleus (fig. 1). Since the generative nucleus resembles the gametic nuclei in structure and staining capacity, it seems probable that in both cases the chromosomes are more contracted and rigid than they are in a typical resting nucleus. When *Bellevia* pollen grains are cultured on agar for several hours, the generative nucleus often divides in the pollen grain. As a rule these divisions are abnormal in two respects: the spindle is curved so that the anaphase chromosomes form a crescent or U-shaped figure, and the chromosomes do not reach their normal degree of contraction (fig. 2). Often the chromosomes are so long that complete separation at anaphase is impossible. Apparently the spindle formation is precocious, so that the nuclear division is initiated before the minor coiling mechanism has effected the normal degree of chromosome shortening characteristic of a normal metaphase.

The bent spindle, as indicated by the behavior of anaphase chromosomes, is also found in occasional abnormal bulges in the pollen tube of *Bellevia* (fig. 3). Evidently the spindle is always curved when space permits, either in the pollen grain or in swollen regions of the pollen tube. In normal pollen tubes the spindle is held within the limits of the narrow tube, and little or no bending of the spindle is possible. These spindles are very long, as indicated by the distance separating the daughter chromosomes at anaphase (fig. 4).

In most mitoses the centromeres of the anaphase chromosomes are oriented toward their respective poles, but in these pollen-tube divisions one or both chromosome arms often pass to the poles in advance of the centric region of the chromo-



FIGS. 1-10.—Camera lucida drawings of generative nuclei divisions from aceto-carmin preparations. Figs. 1-6, *Bellevalia*: 1, chromosomes of generative nucleus in pollen grain; 2, precocious division of generative nucleus in pollen grain showing elongated chromosomes and effect of bent spindle; 3, bent spindle in bulge of pollen tube; 4, anaphase in pollen tube showing wide separation of daughter nuclei and failure of acentric fragments to pass to poles; 5, same showing orientation of free chromosome arms toward one pole; 6, exceptional type of chromosome separation at anaphase. Fig. 7, metaphase in pollen tube of *L. regale*. Fig. 8, one pole at anaphase in pollen tube of *L. regale*. Fig. 9, chromosomes separating at metaphase in pollen tube of *T. paludosa*. Fig. 10, anaphase in pollen tube of *T. paludosa*.

some (figs. 5, 6). In rare cases most or all of the chromosomes at one pole are oriented in the same way as the sister chromosomes at the opposite pole (fig. 5). As a rule the centromeres of an anaphase group of chromosomes are at about the same plane in the cross section of the pollen tube, but occasionally they are scattered along the spindle as in *Lilium* and *Tradescantia* (fig. 6).

Both normal and abnormal division figures were found in pollen tubes of *Lilium regale*, regardless of the technique or the fixatives used. When the tubes were wide the chromosomes tended to become oriented on an equatorial plate at metaphase and to pass to the poles in unison. But in narrow tubes the chromosomes were distributed along the pollen tube at metaphase and were not grouped at anaphase (figs. 7, 8). Such figures were found both in cultured pollen tubes and in those growing in the style, although the cultured tubes were wider and more of the division figures were normal. It is obvious that the chromosomes shown in figure 7 could not possibly form an equatorial plate within the limits of the pollen tube. Nor can this stage be referred to as anaphase, as several critics have done in referring to O'MARA's earlier photographs. Although relational coiling of sister chromatids is found in one chromosome, the chromosomes have reached their normal degree of contraction, as indicated by a comparison of the anaphase figure shown here. Such irregular metaphase figures are common in pollen tubes of *L. regale*, *L. superbum*, and *L. canadense*. Only normal figures were found in cultured pollen tubes of *L. speciosum rubrum*.

No well defined spindle mechanism could be observed in any of our preparations of *Lilium*, although no attempt was made to differentiate such structures with special fixing and staining techniques. A spindle mechanism associated with the centromeres was demonstrated indirectly. Pollen grains were X-rayed and acentric chromosome fragments were produced. At anaphase in the pollen tube such acentric fragments did not pass to the poles but remained in the cytoplasm between the daughter nuclei. Spontaneous acentric fragments in *Bellevia* behave in the same way (fig. 4). The centromere is essential for nuclear division, even in cases where the chromosomes are scattered at metaphase, and where the spindle is not typical. Failure to demonstrate a well-defined spindle presumably is due to unusual cytoplasmic conditions, but under certain conditions of fixation it may be evident (1). According to COOPER, nine of the twelve *Lilium* chromosomes have subterminal fiber constrictions and three have more or less median centromeres. We find ten chromosomes with subterminal centromeres and only two with submedian constrictions (figs. 7, 8).

Pollen tubes of *Tradescantia* grown on agar medium with 8 per cent sugar provide many abnormal division figures of the generative nucleus. Often the chromosomes are distributed along the tube and divide without even approaching an equatorial plate stage (fig. 9). In fact it is difficult to find a typical metaphase plate stage in most of these preparations. Although the chromosomes are not so

well separated as they are following acenaphthene treatment, as described by SWANSON (13), they often are far enough apart so that little overlapping occurs.

These widely distributed chromosomes divide and the daughter chromosomes pass to opposite poles. Such division necessitates the passage of chromosomes in opposite directions at various levels along the spindle. At later anaphase stages the chromosomes are still separated and do not pass to the poles together (fig. 10). Although division and separation of the chromosomes are synchronized, each chromosome appears to behave as an independent unit. Frequently the distal ends of the chromosome arms precede the centric regions toward the poles at anaphase (fig. 10), as is found in both *Lilium* and *Bellevia*. This behavior does not mean that the centromere is inactive at anaphase. The chromosome arms may be unable to turn in the narrow tube, or cytoplasmic streaming may at times carry the distal end of the chromosome to the pole in advance of the centromere. Observations on living material show that the cytoplasm streams rapidly in the pollen tube, although it was not possible to determine the condition of the cytoplasm in the region of the spindle.

The abnormal divisions of the generative nucleus in *Lilium* and *Tradescantia* are related to the nuclear configuration and the spatial limitations in the pollen tube. The generative nucleus in the pollen grain is crescent- or spindle-shaped in longitudinal section. The elongated nucleus passes down the pollen tube, and in late prophase the chromosomes are spaced along the nucleus so that their centromeres may be separated by the length of several chromosomes. Congestion on a metaphase plate is prevented by spatial limitations. When space permits, the chromosomes are able to congregate on an equatorial plate even though rather widely separated at prophase.

The elongated generative nucleus in *Tradescantia* seems to be subject to the stress of cytoplasmic streaming, and it may be divided while in early prophase. In such cases the chromosomes in the two parts of the nucleus may at times develop at different rates, so that one group is at metaphase and the other still in prophase.

When the chromosomes are too widely scattered in the pollen tubes of *Tradescantia* they are unable to divide. They contract and become pycnotic, ultimately disintegrating in the cytoplasm. Apparently a certain degree of association is necessary for the control of division processes and the functioning of the spindle.

#### MECHANICS OF NUCLEAR DIVISION IN POLLEN TUBE

The division of the generative nucleus in the pollen tube may be atypical in several respects. The spindle often is very long and if space permits it may be bent, the chromosomes can divide without the formation of an equatorial plate, anaphase chromosomes can pass to the pole even when widely scattered along the tube, and the distal ends of the chromosome arms may precede the centric region in passing to the pole. Contrary to some of the earlier conclusions, a spindle mech-

anism is operative, although it is not so clearly differentiated as it is in typical somatic divisions. The centromere controls the division and separation of the chromosomes in the division of the generative nucleus as it does in other mitoses.

The long spindle is characteristic of nuclear divisions in the pollen tubes of many species. UPCOTT (15) has suggested that the long spindle is due to the difficulty of chromosome orientation on an equatorial plate. Proper orientation is considered necessary for spindle functioning, and if orientation is delayed the spindle grows until the chromosomes can separate. Apparently spindle formation may not be completely synchronized with chromosome development and division. In *Bellevalia* the chromosomes may be completely contracted and fail to divide because no spindle mechanism is available (fig. 1), or the chromosomes may be separated by a precocious spindle before the chromosomes are contracted sufficiently to permit complete separation at anaphase (fig. 2). It is known that chromosome division, nuclear division, and cell division are independent processes although normally they are harmoniously synchronized (10). The various processes involved in nuclear division also are synchronized in normal mitosis, but those of chromosome development are not always synchronized with spindle formation in the divisions of the generative nucleus, and to a certain extent the two processes are independent. The synchronization of nuclear and cytoplasmic processes is a normal feature of mitosis, but the timing of chromosome development and spindle formation regularly differs in meiosis. This regulated change is responsible for the difference between mitosis and meiosis (2). The synchronization of cellular activities is in accord with OSTERHOUT's suggestion that life is dependent upon a series of processes whose rates bear a definite relation to one another.

The second atypical variation in the division of the nucleus in the pollen tube is the division and separation of the chromosomes without the formation of an equatorial plate. Such behavior is difficult to reconcile with the theory of a repulsion equilibrium which forces the centromeres to lie midway between the poles in an equatorial plate (2); nor can the scattered chromosomes, as shown in figures 7 and 9, be considered as lying on an oblique or buckled plate, as UPCOTT (15) has suggested for less extreme cases in *Tulipa*. It is possible that the great elongation of the spindle, characteristic of pollen-tube divisions, is sufficient to include all the scattered chromosomes and subject them to the spindle influence. Any theory of changes involving centromeres and centrosomes has to account for the behavior of daughter chromosomes at anaphase. A comparison of figures 9 and 10, for example, shows that chromosomes must pass each other on their way to opposite poles. Such behavior cannot be reconciled with a change of equilibrium at the spindle poles, nor can it be attributed to changes in the shape of the spindle due to a repulsion of its polar regions. In these aberrant divisions of the generative nuclei chromosome behavior is synchronized, but each chromosome appears to be capable of

independent division and separation, although all may be under the influence of a common spindle mechanism. In certain respects the apparent independence of the chromosomes in anaphase separation is similar to the independent chromosome spindles in certain coccids, as described by SCHRADER (11).

When the chromosomes become too widely separated in the pollen tube they are unable to divide, suggesting that they must be included within the range of spindle activity to continue normal development.

The third abnormality of the division in the pollen tube is the frequent failure of the centromere to precede the chromosome arms to the poles. The passage of the daughter chromosomes to the poles with the distal ends in advance might appear to be caused by cytoplasmic streaming, but the cytoplasm of the generative cell appears to be homogeneous and unlike the actively streaming cytoplasm of the pollen tube. It is more probable that, if chromosome arms cannot turn in the narrow tube, the force at the centromere simply pushes them toward the pole. These cases, however, are similar to the behavior of anaphase chromosomes in *Sciara* (5), where spatial limitations do not appear to be involved.

It is evident that no type description will fit all nuclear divisions in pollen tubes. In certain genera practically all the divisions may be normal, as we have found in cultured pollen tubes of *Bellevalia*, *Scilla*, and *Zephyranthes*; while other genera, such as *Lilium* and *Tradescantia*, have a high frequency of atypical mitoses. Within a genus some species may have regular divisions and others irregular, as is true in *Lilium*. Even in the same cultures of a given species some pollen-tube mitoses are normal and others atypical. No doubt environmental factors are of importance in determining the type of nuclear divisions, since COOPER (1) found only regular divisions and we find many irregularities in divisions of the generative nucleus of *L. regale*. The effect of environment is most striking in a comparison of pollen tubes of *Narthecium* grown in culture and grown in the styles (18). The cultured tubes are much wider and the division is normal, while those in the style are narrow and no equatorial plate is formed at metaphase. When space permits, the chromosomes pass on an equatorial plate at metaphase and divide regularly at anaphase. When the pollen tube is too narrow to permit free orientation of the chromosomes, they divide without passing on to an equatorial plate and pass irregularly to the poles at anaphase.

### Summary

The type of division found in pollen tubes varies with different genera, species, and among pollen tubes of the same culture. Division of the generative nucleus is generally normal in *Scilla*, *Bellevalia*, and *Zephyranthes*, while atypical divisions are frequent in pollen tubes of *Tradescantia*, *Gasteria*, and *Lilium*. When spatial relations are adequate the chromosomes form on an equatorial plate and the ana-

phase chromosomes pass to their respective poles in unison; but large chromosomes in a narrow pollen tube divide without formation of an equatorial plate, and the daughter chromosomes appear to act as independent units as they pass irregularly to the poles. These divisions are dependent upon the action of the centromeres and a spindle mechanism. Very long spindles are often found in divisions of the generative nucleus, and in *Bellevalia* they are curved or U-shaped when the division occurs in the pollen grain or in bulges in the pollen tube. The relation of these abnormal divisions is discussed in relation to the mechanics of mitosis.

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## CURRENT LITERATURE

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*Geology of Coal.* By OTTO STUTZER. Translated and revised by ADOLPH C. NOÉ. Chicago: University of Chicago Press, 1940. Pp. xiii+461. Figs. 198. \$5.00.

Those interested in the highly varied scientific aspects of coal will find this book extremely useful as a reference. The study of coal has been subject to more than its share of provincialism, and this translation and revision will be of particular value in making facts on coal from European sources generally available.

The subject matter appears to be treated adequately and fairly, although in a few instances opinion may vary. The characterization of Carboniferous plants listed for ecological comparison (p. 169) may include some exceptionable items; the statement that there were no climatic zones in the Carboniferous (p. 172) would seem to need qualification. Paleobotanists will be especially interested in the chapters on coal microscopy, origin of coal beds, and parts of the chapter on stratigraphy of coal deposits. The nature and origin of coal balls is included in the chapter on petrography of coal beds.

Numerous references to the literature are given at the close of each chapter and are a great improvement over the footnote references, often incomplete, given in the earlier German edition. There are few important omissions. The paper by BLACKBURN and TEMPERLEY on the boghead algae (*Trans. Roy. Soc. Edinburgh* 58:841-868, 1936) perhaps deserved mention, since it may be regarded as finally closing the long controversy as to the nature of the boghead algal bodies. Likewise the collection of papers resulting from the two Heerlen Congresses for the Advancement of Carboniferous Stratigraphy might well have been referred to specifically. The older literature seems best represented, but enough references to later work have been included to lead to authoritative works on nearly every topic.

The volume is concluded with an extensive general index, an author index, an index of places, and a list of European and American equivalents of weights, measures, and energy units (the decimal system is used throughout the text). These are of value and to a large extent offset objection of the organization of the book under too few chapter headings. For example, useful minerals associated with coal are treated in the chapter on stratigraphy, although this discussion has no direct bearing on the chapter subject. The complete indices obviate any difficulty in referring to this material, however; in fact, they give assurance of the permanent reference value of the volume.

Personal credit for the excellence of this book is probably not due to any one man. The primary collection of information was accomplished admirably by PROFESSOR STUTZER, but much additional information was incorporated in it by PROFESSOR NOÉ. The death of STUTZER in 1936 and of NOÉ in 1939—while actively engaged in the last of the work of translation—meant that further advice of a technical nature was needed for its successful publication. This service and the tedious work of checking the entire manuscript as a whole, indexing, proofreading, etc., was largely performed by DR. GILBERT H. CADY of the Illinois Geological Survey. His assistance was of inestimable value in completing this task.

The format is excellent, the numerous illustrations generally clear, and the book free from typographical errors.—J. M. SCHOPF.



*The Course of Evolution.* By J. C. WILLIS. Cambridge: University Press; New York: MacMillan Co., 1940. Pp. viii+207. \$3.00.

This volume is a sequence of the author's *Age and Area*, which appeared in 1922 and which questioned the adequacy of natural selection of chance variations as a major factor in evolution. "Evolution and natural selection are probably to a great extent independent, and they work at right angles to one another, with (in plants at any rate) little mutual interference." "Definite single mutations which cause structural alterations, which may, but by no means must, have some functional advantage attached" bring about evolution. The course of evolution is in the downward direction, from family to variety, rather than upward, as required by the theory of natural selection. Evolution moves in the opposite direction to that required by natural selection and is therefore not a matter of chance, but of law which is not as yet understood. Chromosome alterations are thought to be largely responsible for mutations.

The questions of mutation, adaptation, isolation, differentiation, and divergence of variation as factors in evolution are discussed at some length and 34 text cases presented, based upon number of species, morphological and taxonomic characters, and geographical distribution in support of his thesis. The book makes interesting and stimulating reading and is thought provoking, whether or not one agrees with the author.—J. M. BEAL.

*Manual of Cultivated Trees and Shrubs Hardy in North America Exclusive of the Subtropical and Warmer Temperate Regions.* By ALFRED REHDER. New York: MacMillan Co., 1940. Pp. xxx+996. \$10.50.

This second edition of REHDER's excellent treatise needs no recommendation to a public which has used the first one enthusiastically. Introduction of new species and varieties, hybridization, revisions in certain groups as a result of systematic research at the Arnold Arboretum and elsewhere, nomenclatural changes made necessary by the revision of the International Rules in 1930 and 1935, as well as the exhaustion of the first edition, make apparent the need for the present book.

This volume embodies the same general features as the earlier edition, but is approximately 70 pages longer and now includes 2535 fully described and keyed species or hybrids, with about 2685 varieties or lower taxonomic categories, distributed among 486 genera and 113 families. These numbers are in contrast to the 468 genera, 2350 species, and 2465 varieties fully treated in the first edition. In addition, brief treatments of about 25 genera, 1400 species, and 540 hybrids are appended to the units with which they are most closely related. The climatic zone map, based on average annual minimum temperatures, has been revised thoroughly, and now appears enlarged and in two colors as a frontispiece. New data on hardiness assembled in the last 12 years have been incorporated in the species descriptions in accord with the zones now recognized. The list of authors' names has been enlarged; also the glossary, and list of references to illustrations. Family arrangement still follows the ENGLER and PRANTL sequence, with few exceptions, the most important of which is the transfer of the monocotyledons to the end of the book. In some cases generic and specific arrangement has been revised greatly. Double citations of authors' names have been adopted where necessary, in accord with present International Rules. The author has also abandoned his earlier practice of elevating each subdivision of a species to varietal rank, thus avoiding new combinations. In this edition, simple trinomials are used for species subdivisions of any category; and the rank assigned by the cited author, who first placed the subdivisional epithet under the accepted binomial, follows that author's name, even when REHDER does not agree with this rank. He announces his intention to issue a "biblio-

graphical supplement supplying for all the names mentioned in the Manual exact citations of their source and for trinomials the category considered to be botanically correct."—C. E. OLMSTED.

*The Biochemistry of Symbiotic Nitrogen Fixation.* By PERCY W. WILSON. Madison: University of Wisconsin Press, 1940. Pp. xiv+302. Illustrated. \$3.50.

This interesting monograph deals with many of the problems of nitrogen fixation and the difficulties of permanent agriculture due to the gradual transfer of nitrogen from land to sea.

The introductory chapters present the long-range aspects of the nitrogen economy of man and of nature, including the history of legumes in agriculture from Grecian and Roman times to the present. The next chapters take up the chemistry of bacteria; the interaction of host and bacteria; the fixation of nitrogen by bacteria and plant; the carbohydrate-nitrogen relationships in symbiotic nitrogen fixation; the excretion of nitrogenous compounds by legumes; the chemical mechanism of the fixation process; and the physical-chemical characteristics of the enzyme system concerned in fixation. The final chapters consider practical applications of our knowledge of fixation, with some brief concluding remarks on the future of research in the field.

The book is well written, and is worthy of careful reading by anyone who wishes a comprehensive summary of the entire field of nitrogen fixation by bacteria and legumes. The 32 pages of literature citations will be useful in obtaining more complete treatment of particular phases. There are 34 full-page plates and 27 text figures. A short subject index adds to the usefulness of this valuable treatise.—C. A. SHULL.

*If They Could Speak.* 120 Broadway, New York: Chilean Nitrate Educational Bureau, 1941. Pp. 54.

This is an attractive handbook of the mineral deficiency diseases of plants. The entire story is told in colored reproductions made from Kodachrome transparencies—95 of them—taken in the field, with brief descriptive identifications accompanying the plates. The deficiencies included are of boron, calcium, copper, iron, magnesium, manganese, nitrogen, potash, and zinc. About 35 species of plants are portrayed, including many important crop plants, such as alfalfa, corn, tobacco, soybean, wheat, etc.

The Chilean Nitrate Educational Bureau has a fine conception of its opportunities for service, and this handbook is additional evidence of enlightened policy. Copies may be obtained merely by requesting them. The director of the Bureau, MR. HERBERT C. BREWER, would welcome comments on the reproductions from anyone who has first-hand knowledge of deficiency diseases of plants.—C. A. SHULL.

*The Ferns and Fern Allies of Wisconsin.* By R. M. TRYON, JR., N. C. FASSETT, D. W. DUNLOP, and M. E. DIEMER. Madison, Wisconsin: Department of Botany, University of Wisconsin, 1940. Pp. v+158. Figs. 215; 76 distribution maps. \$1.00.

Combining excellent photographs of natural habitats and of herbarium plants, low magnification photomicrographs, drawings of leaves and spore-bearing structures, and distribution maps for nearly every species and variety growing in Wisconsin, this manual has achieved a new standard of excellence. The nomenclature follows the latest International Rules, with the often varying synonyms of GRAY's Manual and BRITTON and BROWN's Flora parenthetically included. The key to the genera and the key to species of ferns based on sterile leaves (which is well illustrated with labeled drawings and photographs) will satisfy the professional botanist and the

interested amateur. The description of each plant is short and critical. The distribution for Wisconsin and for other states and countries is given.—P. D. VOTH.

*Moss Flora of North America North of Mexico*. Vol. II, Part 4. By A. J. GROUT. Newfane, Vermont: Published by the author, 1940. Pp. 211+285. Illustrated. \$2.50.

DR. A. LEROY ANDREWS treats the genera *Bryum* and *Rhodobryum*, the Mniaceae, and the Rhizogoniaceae in this concluding part of the flora. The successful treatment of the difficult genus *Bryum*, together with additional notes on the Bryaceae, an artificial key to *Pohlia* in the form of an appendix (by GROUT), as well as additions and corrections to all three volumes, make this last part especially valuable. Many of the plates were drawn or supplemented by SEVILLE FLOWERS.

Requiring 12 years for its completion (see previous reviews in BOT. GAZ. 88:III, 1929; 93:III, 1932; 96:578, 1935; 101:509, 1939), and having the cooperation of America's most successful amateur and professional bryologists, this flora supplies a great need in this hemisphere. State bryological floras will now have a basis for greater unity; professional bryologists will be saved endless effort; and, it is hoped, amateurs will be encouraged to begin the study of plants in general and of the mosses in particular.—P. D. VOTH.

*Mechanisms of Biological Oxidations*. By DAVID E. GREEN. New York: Cambridge University Press, 1940. Pp. 181. \$2.75.

The oxidizing enzymes have probably been more intensively investigated in recent years than any other group. This book gives a detailed account of the properties, chemical nature, factors affecting and mode of action of those oxidizing enzymes whose prosthetic group contains respectively copper, iron porphyrin, zinc, flavin, nicotinic amide, thiamine, and glutathione. Certain enzymes whose prosthetic group has not yet been isolated are also treated. Wherever known, the steps in the oxidation of a compound are given, with the exact mode of action of the enzyme concerned. As the book shows, considerable progress has been made in putting the oxidation processes of the living organism on a purely chemical basis. Also the studies show some relationships between enzymes on the one hand and vitamins and trace metals on the other.

Biologists will welcome this critical summary of the literature of the oxidizing enzymes.—S. V. EATON.

# THE BOTANICAL GAZETTE

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## LIFE HISTORY OF PELTANDRA VIRGINICA

BENJAMIN GOLDBERG

(WITH FORTY-NINE FIGURES)

### Introduction

A morphological study of *Peltandra virginica* Kunth was made to assemble data which would give a rather complete life history of a widespread plant and a basis for comparison within and outside the Araceae. Features neglected or incompletely ascertained in the plant and the family as a whole were studied as fully as possible.

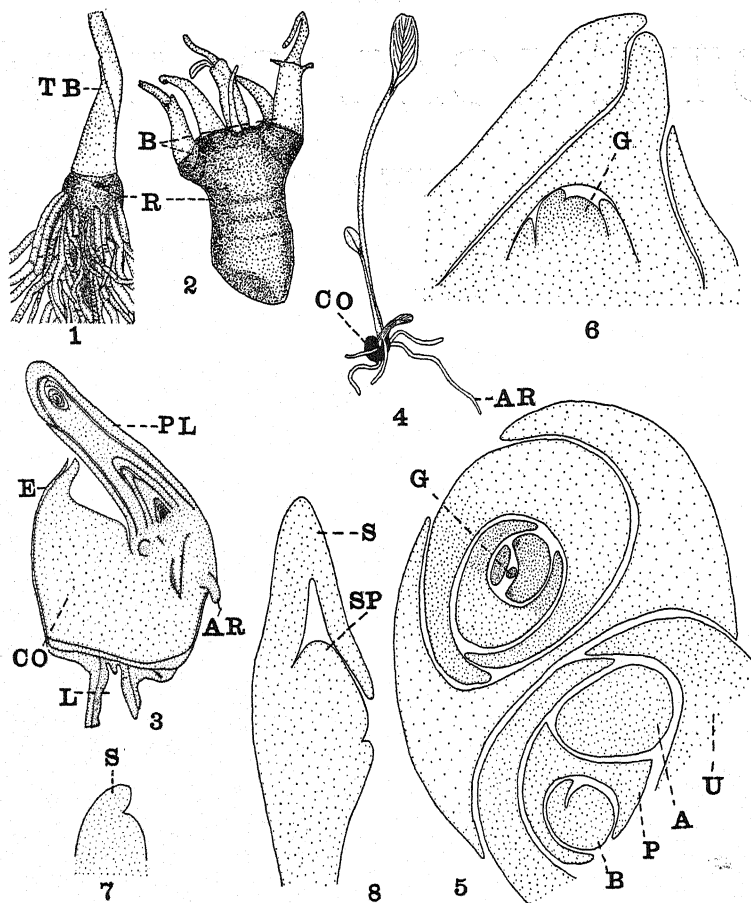
Comparative studies on the aroids (13, 23) have been of interest since ENGLER (10, 11, 12) pointed out that in spite of appreciable variation there were unifying tendencies in the group. The only detailed morphological account of *Peltandra* (7) deals with part of the development of the pollen. Additional reports include a microchemical study of the seed and its germination (18), a demonstration that the seed can germinate in almost total absence of oxygen (8), and an account of seed frequencies (9).

### Observations

*Peltandra* is found throughout the eastern United States and has been reported west of the Mississippi River. It is an inhabitant of fresh water marshes, especially along the banks of tidal rivers. The stem is a subterranean vertical rhizome (figs. 1, 2R), cylindrical, and with a diameter in mature, unbranched specimens of 8 cm. The leaves are so compactly arranged that internodes appear nonexistent. The rhizome sometimes branches (fig. 2B) and the branches become separated from the parent plant as the basal portion of the rhizome decays. Since branches as well as parent axis form erect rhizomes, there results characteristically a cluster of plants.

Some rhizomes bear 100 or more adventive roots, which reach a diameter of

about 7 mm. in their proximal portions. Roots arise acropetally on the rhizome (fig. 1) and sometimes pierce the sheathing leaf bases. Wrinkling of the outer cortical tissues of roots of seedlings and older plants occurs, sometimes contracting to almost 50 per cent of the original length in some regions.



FIGS. 1-8.—Fig. 1, plant collected in October. Fig. 2, branched plant in dormant state with roots removed. Fig. 3, longitudinal section of germinating seed (*CO*, cotyledon; *L*, lumen of haustorial cell). Fig. 4, seedling 16 days old with cotyledon (*CO*) still within pericarp. Figs. 5, 6, transverse and longitudinal sections through apex of mature rhizome. Figs. 7, 8, longitudinal sections of young inflorescences.

Above the concave top of the rhizome during most of the year is a large conical terminal bud (fig. 1 *TB*) about 15 cm. long, extending from the top of the rhizome to the soil surface. From this bud, containing rudiments 15 cm. to 50  $\mu$  long, only a part of the outer, older, and larger rudiments emerge in late spring; the remaining ones, excluding those which abort during the interim, emerge during the fol-

lowing year. The various regions of the leaf are clearly defined. Leaves of mature form and size have a sheath 4.5–6.5 dm. long (including the subterranean portion extending from the soil surface to the apex of the rhizome), a stalk 2–4 dm. long, and a sagittate to hastate lamina reaching at times a length of 5 dm. (from the acuminate apex to the rounded basal lobes) and a width of 2 dm.

The larger inflorescences are about 70 cm. long, from the subterranean proximal region of attachment of the peduncle to the distal end of the spathe. The dark green spathe is 20–30 cm. long and extends a few centimeters beyond the inclosed spadix. While there is much variation, most plants in Maryland are at the height of their vegetative and flowering activity by the end of May or early June. After pollination, which occurs at this time, the distal part of the spadix, covered with staminate flowers, disintegrates, together with the distal part of the spathe, marked off by a slight invagination. The erect inflorescences bend below the attachment of the spathe to the peduncle, the fruiting spadix finally pointing toward or resting on the soil. The fruits, surrounded by the enlarged basal part of the spathe, ripen in August or September.

#### SEED AND SEEDLING

The dark green to brown obovoid fruits measure about 1.5 cm. long and 1 cm. wide, and show an apical scar marking the former position of the style. Within the membranous pericarp lies usually one, but sometimes as many as three, seeds imbedded in a colorless jelly-like material.

To the conspicuous, flattened-globular, micropylar portion of the seed is attached an obovoid chalazal appendage about 2–2.5 mm. long, which marks the position of the large haustorial cell of the endosperm. Within the seed lies the large embryonic sporophyte enveloped by the residual endosperm and the almost entirely withered parent sporophytic tissue. The layer of remaining endosperm surrounding the embryo is one cell thick, except in the region nearest the empty haustorial cell, where it is about seven cells thick. The integuments have become brownish and shriveled, but the parent sporophytic tissue in the chalazal region forms a thick coat around the empty haustorial cell.

The pale green cotyledon forms the greater part of the embryo, and has almost the same size and shape as the more conspicuous micropylar portion of the seed. In a deep groove of the cotyledon near the apex of the seed lies the narrowly conical, green plumule, about 9 mm. long and 3.5 mm. in diameter at its base. It contains six or seven foliar structures. Since it is partly encircled by the upgrowth of cotyledonary tissue, it forms with the cotyledon a compact body. The radicle does not form a group of tissues topographically delimited from the surface of the rest of the embryo. Occasionally the hypocotyledonary portion of the radicle appears on the side of the cotyledon as a poorly defined swelling, 1.5 mm. thick and 4–5

mm. wide. About 1.5 mm. below the base of the plumule on its exposed side lies the primary root, a slight rounded protuberance less than 1 mm. in diameter. Around the primary root the surface of the hypocotyl is marked by three to five small, circular, partially hyaline areas which indicate the position of adventive root primordia.

Usually the seeds germinate in April, but in some cases they germinate in the late summer of the year the ovules were fertilized, and while the fruits are still attached to the parent plant. By the time germination occurs the ovary wall is usually so decayed that it presents no mechanical obstacle to germination. As the plumule rises from its cotyledonary groove (fig. 3 $PL$ ), the membranous envelope ( $E$ ), consisting of the remains of endosperm and integuments, is ruptured. Although the primary root generally fails to elongate, it may attain a length of 2.5 cm. before withering. The adventive roots (fig. 3 $AR$ ) grow and elongate rapidly; the first ones are small and short-lived, those formed later approach by degrees the mature size. Figure 4 shows a seedling, with a few adventive roots ( $AR$ ) and the first three cauline foliar structures, 16 days after germination began. The first emergence was a bladeless sheathlike structure about 2 cm. long, the second and third possessed elliptical laminae, and the third alone a distinct stalk below the lamina. The primary axis increases rapidly in diameter, so that the young rhizomes show an obconical tapering.

#### MATURE PLANT

BRANCHING.—The seedling behaves as a monopodium until initiation of the first inflorescence, which is developed directly from the apex of the primary axis. Apical growth is then carried on by a bud which develops in the axil of the penultimate leaf. In the axil of the ultimate leaf a second bud is formed, which gives rise only to a basal acroscopic, two-keeled prophyll and a second inflorescence at its apex. The bud in the axil of the penultimate leaf completely displaces the original apex of the shoot in prominence and central position, so that outwardly the branching system appears unchanged, and the branch developing from this bud also ceases its apical growth with the formation of an inflorescence, after it has given rise to its share of leaves. Apical growth of the plant is then maintained by a bud in the axil of the penultimate leaf of the branch. The process is continued; and though a mature plant has an erect vertical rhizome, it is actually composed of superposed branches of increasingly higher rank. ENGLER (10), recognizing the sympodial nature of the shoot in practically all the Araceae, regarded the shoot corresponding to the one arising from the bud in the axil of the penultimate leaf of the *Peltandra* shoot as a unit in the structure of the plant and called it a *Fortsetzungspross* (continuation shoot).

As in the primary axis of the seedling, a second inflorescence (fig. 5 $B$ ), together with a two-keeled prophyll ( $P$ ), is borne in the axil of the ultimate leaf ( $U$ ) of each

continuation shoot. In several hundred plants examined in 5 years, inflorescences were never observed in other positions on the continuation shoot. The second, or lateral, inflorescence originates after the first (fig. 5A) and always lags behind it in development. Both inflorescences of a continuation shoot, because of their position in initiation and development, are enveloped by the ultimate leaf.

Frequently the antepenultimate leaf of a continuation shoot bears an axillary bud, the *Vermehrungsspross* (12) or vegetative bud (28). In a series of plants collected in 1939, the vegetative bud was found on 118 out of 188 continuation shoots. Some plants bear no vegetative buds whatever; others show them only in part; and still others bear one in the axil of every antepenultimate leaf or leaf rudiment. Vegetative buds, if they develop at all, result in the branching of the rhizome and subsequent vegetative multiplication of the plant. In *Symplocarpus* and *Lysichiton* (28) the vegetative buds occur in a definite position, that is, in the axil of the lowest leaf of the continuation shoot, but do not develop into branch shoots. In *Peltandra* the buds often do not develop, but many do, some after lying dormant for a time and after the surrounding organs have emerged and disintegrated.

In a series of plants collected from 1937 to 1939, 207 out of 300 continuation shoots showed vegetative buds in the axils of the antepenultimate leaves; two of the 207 also showed them in the axils of the ante-antepenultimate leaf. None were observed in the axils of older leaves, except on the primary axes of seedlings. In one such case thirteen vegetative buds were found, one in the axil of each of thirteen leaves. Vegetative buds were never observed in the axils of the ultimate or penultimate leaves of a shoot.

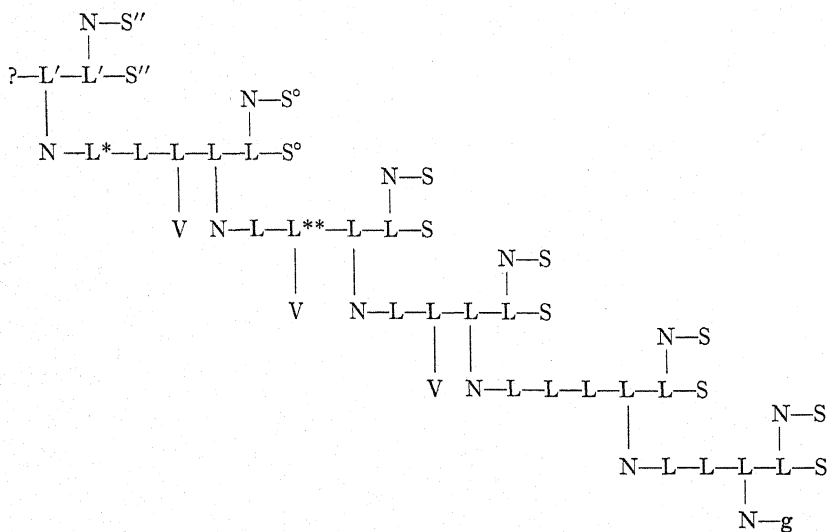
Morphologically both the vegetative bud and the continuation shoot arise as buds of equal rank, but they differ in appearance and composition as well as in development. Both start with two-keeled, acroscopic prophylls. On the continuation shoot the next foliar structure is a leaf with a lamina of mature form and size; on the vegetative bud one or two more bladeless leaves are borne, while the laminae on subsequent leaves attain only by degrees the mature form and size. The vegetative bud is flattened dorsiventrally much more than the bud of the continuation shoot. After initiation, development of the bud of a continuation shoot is rapid, while that of the vegetative shoot is much slower and sometimes even appears to cease.

NUMBER OF LEAVES PER CONTINUATION SHOOT.—ENGLER pointed out that the number of laminated structures appearing in a growing season, or on a continuation shoot, is often limited in the Araceae, only one being formed in species of *Amorphophallus* and *Philodendron*. In *Peltandra* the number in general decreases with increase in size and with maturity of the plant. On the other hand, branching of the rhizome, through development of vegetative buds, is apparently associated with an increased number of foliar structures on a continuation shoot.

The minimum number of foliage leaves found on a continuation shoot was three.



The following diagram is based on ENGLER's method of summarizing shoot structure types in the Araceae (10), and has been modified to represent the complete record of a single specimen at the height of its activity. A continuation shoot and a vegetative bud of equal rank are represented by one horizontal row, and each is connected by a vertical line with the leaf in whose axil it is borne. Lateral inflorescence branches are similarly connected by vertical lines, but placed above their parent axes for clearness. Horizontal lines connect organs at successively higher levels, as read from left to right, on the same shoot.



ESTIMATED AGE OF RUDIMENTS AT TIME OF EMERGENCE.—Characteristics of mature plants such as that diagramed may be enumerated as (*a*) small number of leaves (3-5) on a continuation shoot; (*b*) large number of inflorescences for time of year in which examined; (*c*) mature form and large size of leaf (not so reliable an index as the others); (*d*) unbranched rhizome with large diameter; and (*e*) vegetative buds either not initiated or arrested in development after attaining position

for emergence of rudiments. Plants selected by these criteria as mature agreed, to a certain extent, in number of leaf and inflorescence rudiments contained in the terminal bud during the dormant season, in number of organs emerging during the growing season, and in number of primordia added to the bud in summer by activity of growing points of successive continuation shoots. In view of this uniformity, periodic examinations of this type of specimen should reveal, within limits, details of sequence of the emergence of rudiments and their initiation, and the time elapsed from formation to emergence of a leaf or an inflorescence. The numerical range of leaves and inflorescences in mature, rudimentary, and aborted condition

TABLE 1

NUMBER OF LEAVES AND INFLORESCENCES FOUND IN MATURE, RUDIMENTARY, AND ABORTED CONDITION ON MATURE PLANTS THROUGHOUT THE YEAR

MONTH	CONDITION OF PLANT	SPADICES IN BUD	LEAVES IN BUD	SPADICES IN FLOWER OR FRUIT	EMERGED LEAVES	ABORTED SPADICES	LEAVES PER CONTINUATION SHOOT
Jan.....	Dormant	9-10	.....	0	0	0-1	.....
Feb.....	Dormant	10	.....	0	0	0-2	4
Apr.....	Dormant	10-11	.....	0	0	0-1	.....
May.....	Dormant	10-12	20-25	0	0	0-2	3-5
June.....	In flower, fruit, and foliage	6-8 (10)*	15-18	3-5 (6)†	6-8	0-1	3-5
July.....	In foliage and fruit	7-9	17-21	3-5	5-9	0-1	3-5
Aug.....	In foliage and fruit	8-11	21-25	2-4	5-6	0-2 (3)†	3-5
Sept.....	Dormant	10-11	ca. 23	0	0	0-2	4-5
Oct.....	Dormant	10-12	20-22	0	0	0-2	3-5

\* Only one specimen found with ten rudimentary inflorescences at this period; the two oldest seemed about to flower.

† Only case of its kind found.

found on mature plants at various times during the year is shown in table 1. In an average mature specimen, five to nine of the twenty-odd leaves in the terminal bud at the beginning of May, together with three or four of the ten to twelve inflorescences present, emerge by the early part of the summer. Usually one to three leaves and not more than two inflorescence rudiments (the oldest and outermost of the terminal bud) abort between yearly emergences, and are found outside the terminal bud after the yearly crop of organs has emerged and degenerated. Thus about half the rudiments in a terminal bud early in May remain intact in the bud during the subsequent summer and dormant season.

Rudiments initiated during the summer (July-October) are added to those already present in the terminal bud, while the latter increase in size and assume the relative positions occupied by the recently emerged organs before emergence. By October, formation of new rudiments has apparently ceased, since the number re-

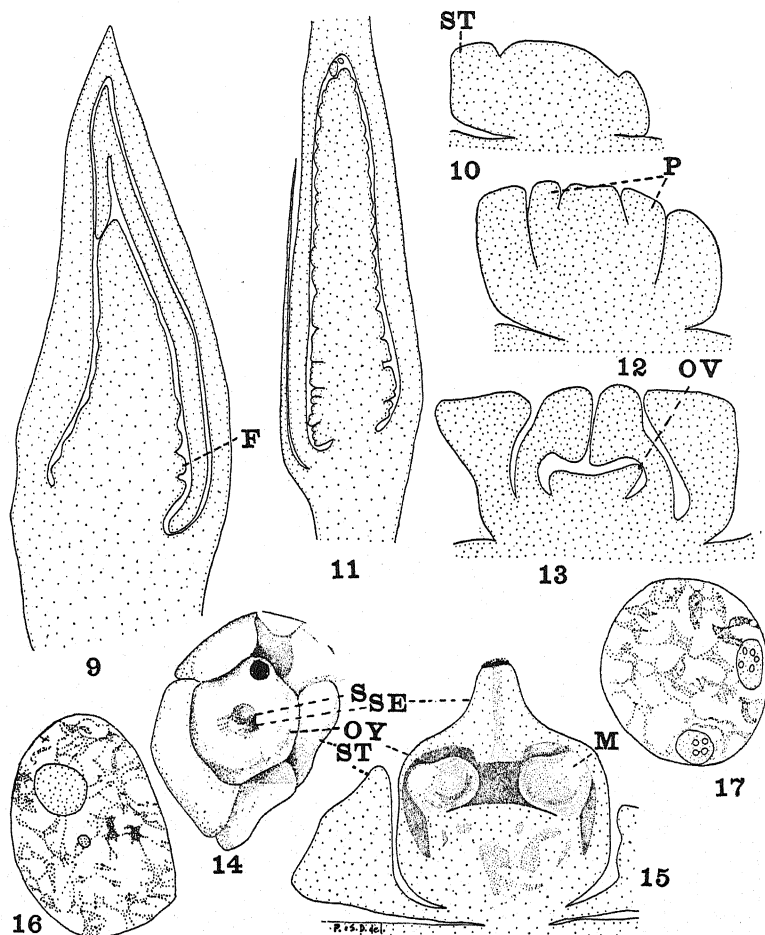
mains the same from October to May and no abortions were observed during this period. It appears that rudiments initiated prior to October of one year do not emerge until about 20 months later (cf. *Symplocarpus* 27), shortly before the second succeeding summer.

**APEX OF SHOOT AND ORGAN INITIATION.**—The growing point of the rhizome and the youngest organ primordia lie in a slight apical depression, at the center of which the growing point of the youngest continuation shoot (figs. 5, 6, *G*) can be seen as a dome-shaped structure, overarched and enveloped by leaf primordia. First indications of a leaf are periclinal divisions in the hypodermal layer of the apical region. Very early the tip of the young primordium is marked off by its less meristematic appearance. In the young leaf it is disproportionately large as compared with its relatively insignificant size (about 5 mm.) in the mature organ.

The spathe appears to originate just as a leaf does, although a detailed study of this was not made. Its nature is soon revealed by failure to show the adaxial thickening that characterizes the early stages of petiole-midrib development, by elongation of the axis below its insertion in the formation of the peduncle, and by its position relative to the rapidly developing bud in the axil of the penultimate leaf. If a vegetative bud is to be formed in the axil of the antepenultimate leaf, it appears about the same time as the bud which gives rise to the new continuation shoot. The bud which gives rise to the lateral inflorescence is initiated later. Like the leaf, the spathe early surpasses and envelops the former growing point (figs. 7, 8, *S*) and forms a conspicuous tip, although it develops neither a stalk nor a blade. As the axis below the insertion of the spathe elongates, the former shoot apex develops directly into the spadix (fig. 8*SP*), the outline of which soon becomes slightly undulate. The number of swellings increases acropetally as those more basally situated become pronounced and flattened (fig. 9*F*).

As a result of continued distal flattening, the floral primordia appear in section as inverted truncate wedges. The primordia on the basal part of the spadix give rise to lateral members (fig. 10*ST*), appearing usually as incomplete or infrequently as complete rings, which mark the beginning of the staminodes. Later the primordia give rise to a ringlike lateral growth just within the staminodes (figs. 11, 12*P*). At first the staminodes, the newly formed ring, and the central truncate portion of the primordium develop at about the same rate. Subsequently the ring surpasses the other elements, curving centripetally as it envelops the central region. The edges of the ring, as they grow toward each other, flatten in a direction perpendicular to the long axis of the spadix and form the narrow, slitlike, stylar canal (fig. 13). The proximal part of the former ringlike structure extends centrifugally (fig. 13), forming the ovary; and the central truncate part of the floral primordium becomes the flattened basal placenta which gives rise to the ovules at points around its edge (fig. 13*OV*).

The more apical and by far the larger number of floral primordia do not give rise to lateral members (fig. 11); the flattened protuberances merely increase in bulk. Eventually these primordia, which give rise to the staminate flowers, be-



FIGS. 9-17.—Figs. 9, 11, longisections of young inflorescences. Figs. 10, 12, 13, same of young pistillate flowers. Fig. 14, mature pistillate flower from above. Fig. 15, longisection of same. Figs. 16, 17, early meiotic stages in microsporocyte nuclei.

come roughly rhomboidal to hexagonal when viewed in a section tangential to the surface of the spadix. Undulations arise along the edges of these staminate flowers and mark the origin of individual microsporangia, which usually occur in pairs. As development proceeds, adjacent pairs of microsporangia often become separated by sharp invaginations. This compact structure, bearing usually

sixteen to twenty microsporangia and representing a staminate flower, is the synandrium.

While the spadix may bear staminate flowers almost to the tip, sometimes its distal end is sterile. The synandria are usually flat-topped but sometimes show centrally located depressions of varying extent, which on evidence from transition forms are regions where pistils would be located.

Both ENGLER and BENTHAM and HOOKER described the staminodes as fused into a ring around the ovary. While this condition has been found, more often one to five separate staminodes to a pistillate flower were noted (fig. 14ST). Sometimes the staminodes were so large as completely to fill the spaces between pistillate flowers; at other times they were so inconspicuous as to be covered by the bulging sides of the pistils, and sterile regions of the spadix were then visible between the flowers. Rarely the staminodes of the more distal pistillate flowers bear apparently normal pollen. Staminate flowers may abut directly on the pistillate, but sometimes a transition zone is indicated by relatively few and scattered flowers, or flowers consisting exclusively of staminodes.

The normal pistil (figs. 14, 15) has a slightly flattened globular ovary (OY) with a short style (SE) and a terminal stigma (S), but various types of incompletely developed pistils occur between staminate and pistillate parts of the spadix. In addition to ovules borne normally on the placenta, they were sometimes borne at the base of the style on the protuberance into the ovarian chamber below the top of the ovary. Six substylar ovules were found in an ovary which contained also seven placental ovules.

#### DEVELOPMENT OF POLLEN

DUGGAR's observations (7) on development of the pollen in *Peltandra* did not cover some of the earlier stages of meiosis, and the writer differs with him on some interpretations. As noted elsewhere (6), the chromosome number 22 given by DUGGAR does not appear to refer expressly to either *Peltandra* or *Symplocarpus*.

When preparations made from staminate flowers fixed without previous dissection showed the synizetic phenomenon in the earlier stages of meiosis, it was found necessary to dissect out contents of individual microsporangia in the fixing fluids in order to avoid clumping of the nuclear material. Sporocytes fixed in this way for temporary aceto-carmin mounts, or for imbedding and sectioning by the alcohol-xylol-paraffin method, gave about the same results as fresh untreated sporocytes dissected and mounted in paraffin oil. While it is evident that artifacts may be produced in the material during dissection, and indeed the instances of cytomixis observed are probably the result of tension and pressure, yet the frequent absence of distortion in all the stages, and another aspect of nuclear behavior to be discussed later, encourage confidence in the results obtained.

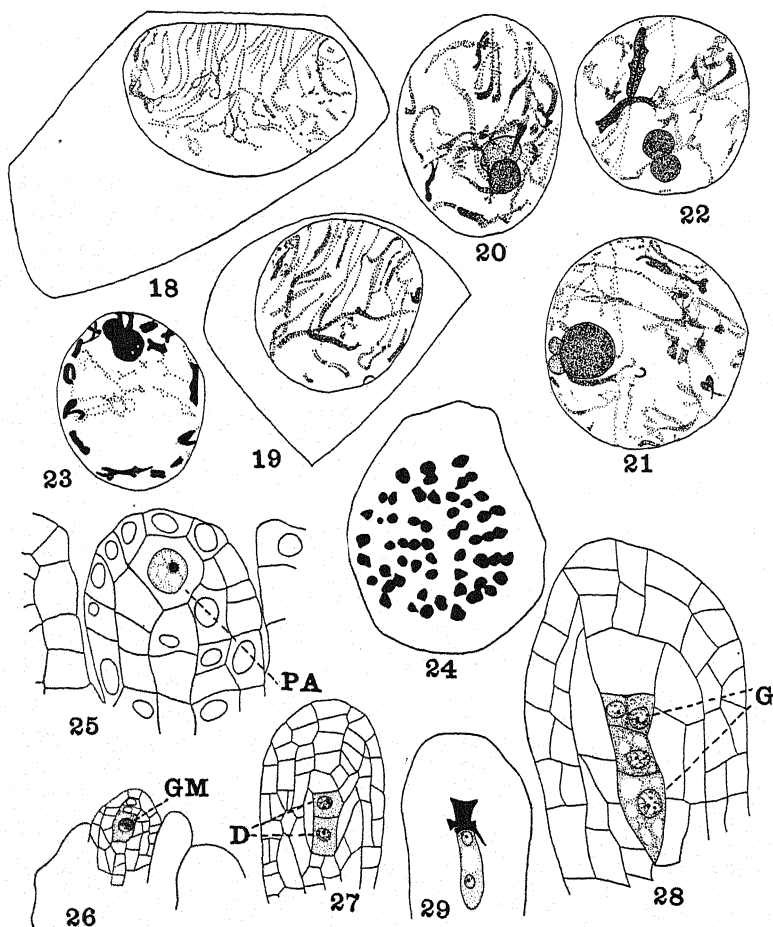
In the microsporangium, as in the ovule, the definitive sporogenous tissue is not marked off until the spring in which the meiotic divisions and flowering occur. Within a month of the occurrence of meiosis, the tapetal, wall, and sporogenous layers can be distinguished. Somatic mitoses occur in the sporogenous cells during the same season that the meiotic divisions occur. The wall is four to seven cells thick, the tapetum two to three, and the sporogenous region four or five in cross section. At length the cells of the sporogenous tissue enlarge, containing larger nuclei and denser cytoplasm than cells of surrounding tissues.

At first the large resting nuclei of the microsporocytes contain four or more nucleoli, stain lightly with Heidenhain's haematoxylin, and appear to contain fine filamentous structures with scattered, more chromatic material (fig. 16). As the nuclei enter the meiotic prophase, bodies similar to prochromosomes are formed; that is, vaguely limited bodies, roughly rectangular in section, and composed—in part at least—of filamentous structures (fig. 17). The delicate chromonemata observed in early meiosis seem to arise from the prochromosome bodies, and later stages show their gradual evolution and the initiation of their orientation. The few denser-staining regions evident in the resting nucleus give way to scattered, darkly staining structures which seem to become incorporated with the fine threads. The nucleoli tend to fuse in pairs. At this point, while dissected material showed a polarization of the threads, other material showed the familiar synizetic ball. Eventually a majority or all of the threads become polarized ("bouquet" stage) and extend parallel to each other from one side of the nucleus. Toward the periphery of the nucleus the chromonemata curve outward. The threads at this stage do not stain deeply and no structure could be discerned inside them, but their arrangement (fig. 18) indicates that they may be pairing. Sometimes there is a definitely thicker strand, which may be an already synapsed thread. More definite conclusions concerning behavior at pairing cannot be formed as yet.

In preparations showing polarization of the chromonemata, orientation of the threads in different nuclei was more or less random, except that frequently in two neighboring nuclei the chromonemata were oriented toward each other, perpendicular to a common cell wall separating them. This tends to emphasize the validity of the method of fixation, since it suggests that the chromonemata may be oriented with respect to the last somatic metaphase plate. The random orientation otherwise observed in any sporogenous mass seems to invalidate the suggestion that the arrangement is the result of an external mechanical disturbance, since if the latter were true, orientation of chromonemata in different nuclei would tend to be uniform and the nuclei otherwise distorted. A similar bouquet stage has been observed in various animals and plants (4, 5, 14, 19, 24, 31, 32).

The next stage was frequently observed. The threads, thicker in diameter and

staining more readily and sharply, still show in part the polarized arrangement of the bouquet. Clearly they are fewer in number. Their dual nature is sometimes obvious (fig. 19). The chromonemata do not appear homogeneous but are composed of densely staining granules (chromomeres) separated by less chromatic



FIGS. 18-29.—Figs. 18-24, stages in meiosis in microsporocytes. Fig. 24, polar view of metaphase. Figs. 25-29, longisections of nucellus or distal portion of ovule, showing development of female gametophyte.

regions. Although such bodies were not found in general, their presence was clear in a few cases. The chromonemata at this stage did not show uniform diameter throughout their length. This phenomenon, called first "partial contraction" and more recently "differential condensation," has been noted in animals and plants (4). In *Peltandra*, as in some other angiosperms, it seems to persist from

this late zygotene stage up to the beginning of diakinesis (figs. 19-23). From this time on the nucleoli are usually fused into one body, or closely associated, and contain few to many vacuolar spaces. With this, zygotene is complete and pachytene is gradually entered. It is clear from the number of ends evident in the ensuing stages that the chromonema is not a continuous thread, although the number of elements could not be ascertained. As the chromonemata grow shorter, the so-called spireme stage is reached. Material used for study of this and subsequent stages was not fixed with the same precaution for most rapid penetration as material for preceding stages; hence observations will be reported briefly.

The last traces of polarization vanish and the threads extend throughout the nucleus as the spireme or pachytene stage progresses. Following pachyphase, the chromonemata begin to show marked differences in chromaticity in some regions (figs. 20-22), and as they shorten tend to come into contact. These phenomena are more marked through the diplotene stage. With advanced diplotene (fig. 21) the contracting chromonemata migrate toward the periphery of the nucleus, and their dual nature is plain (fig. 22). Characteristic tetrad shapes appear (fig. 23).

After disappearance of the nuclear membrane the spindle, at first multipolar, becomes bipolar (cf. 7). The haploid number of chromosomes, as ascertained by the various stages of the heterotypic (fig. 24) and homoeotypic divisions, is 56. The meiotic divisions are "successive." Localization of chromatic material never entirely disappears from the chromosomes at interkinesis, although irregularly outlined nucleoli, one or more per dyad, appear.

As the tetrads of microspores become established, the walls of the tapetal cells disappear and their cytoplasm appears to migrate throughout the locule and to separate the tetrads. Later the nuclei of the tapetal cells leave their peripheral position and become distributed throughout the microsporangium. The individual microspores become rounded off and invested with a heavy, at first unsculptured, wall. The periplasmodial nuclei are amoeboid in form. At maturity, just before the pollen grains are shed, they measure about  $25\mu$  in diameter, are sculptured with short, pointed spines, and contain a large nucleolated pollen tube nucleus and two smaller (male) nuclei devoid of nucleoli and staining densely. The wall of the mature pollen grain is pitted. The pollen-tube nucleus, which becomes lightly staining, irregular in outline, and apparently degenerate, may precede or follow the male nuclei into the pollen tube. Tests showed abundance of starch grains in mature pollen grains and pollen tubes. The male nuclei usually have surrounding hyaline areas clearly delimited from the granular cytoplasm of the tubes (cf. 29). The possibility that these hyaline areas are artifacts has not been overlooked. Sometimes a distinct granular cytoplasmic layer can be seen surrounding each male nucleus, and this in turn is surrounded by the clear areas.

At maturity the sessile, staminate flowers consist of an angular disk 3-5 mm.



long, 2–3 mm. wide, and about 1 mm. high. In a tangential section of the spadix a sharp cusp appears between the upper portions of the sporangia of each of the pairs arranged around the periphery of the floral disk. This cusp marks the sole region in which the cells of the hypodermal layer of the theca remain small, with walls unthickened. The one-cell layer envelope of mechanically strengthened cells also occurs on the side of the loculus attached to the floral disk. As the periplasmodium of each microsporangium and the septum separating the microsporangia of each theca disappear in the course of development, the pollen masses become confluent. The wall of the theca ruptures in the region of the unstrengthened hypodermal cells. At first the freshly shed pollen tends to remain in vermiform masses near its place of exit on the outer surface of the synandrium, but on being disturbed most of it falls into the dilated proximal part of the spathe.

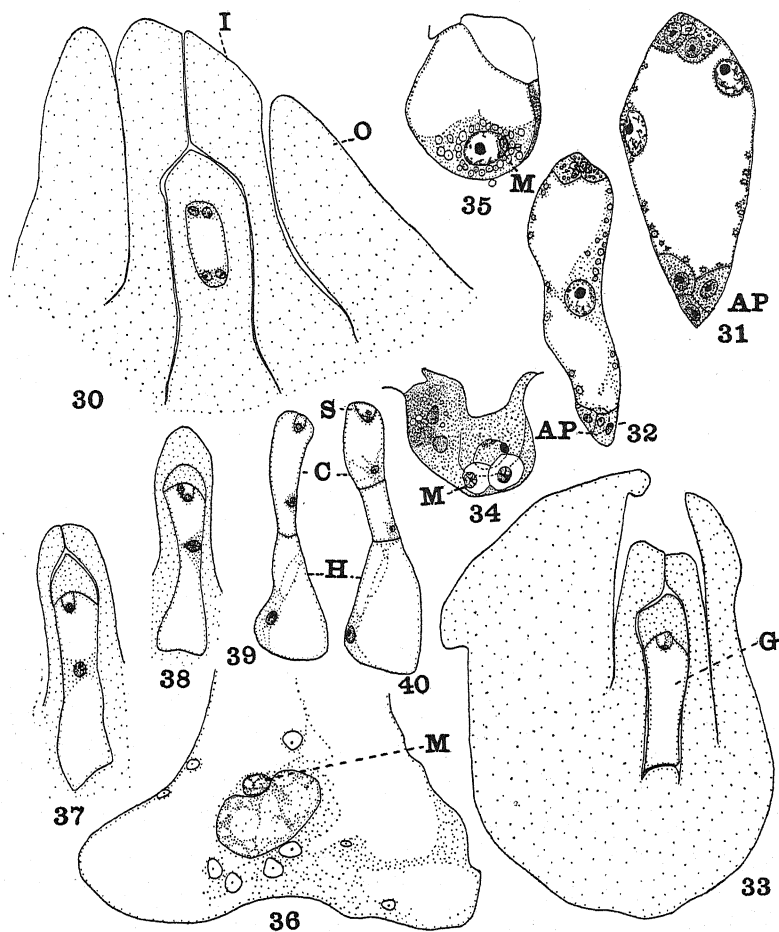
#### DEVELOPMENT OF MEGAGAMETOPHYTE

The primordia from which the one to ten ovules arise grow upward and outward from the edge of the basal placenta and eventually approach the wall of the ovary in its upper regions. At the first sign of the integuments, the primary archesporial cell is not clearly marked off. The inner integument differentiates more rapidly than the outer. A median hypodermal cell of the nucellus is differentiated as the primary archesporial cell (fig. 25*PA*). The megaspore mother cell (fig. 26*GM*) can be seen with two overlying tapetal cells, which were probably formed by division of the sister cell of the megaspore mother cell. *Symplocarpus* (27) and *Calla* (23) also show tapetal cells, but other aroids—for example, *Acorus calamus* (1)—form none. Throughout development of the ovule, the portion of the outer integument on the side next to the funiculus is larger than that diametrically opposite. The micropylar end of the hemianatropous ovules is consistently directed outward toward the wall of the ovary (fig. 15*M*).

By the time the first meiotic division has occurred the megaspore mother cell is well surrounded by nucellar tissue, and the distal part of the inner integument is growing contripetally and overarching the nucellus, forming the narrow micropyle. At first the outer integument does not keep pace in growth longitudinally with the inner, but it is broader than the inner integument, which is two or three cells thick, except at its tip. As in other aroids (23), the inner layer of the inner integument forms a distinct layer of cells, rectangular in outline, with their longer axes perpendicular to the longitudinal axis of the ovule (the “tapetal” layer of the megagametophyte).

The spindle in the smaller, micropylar member of the dyads (fig. 27*D*) is perpendicular to, and the spindle in the chalazal dyad (fig. 28) is parallel to, the longitudinal axis of the ovule. Of the four megasporos formed (fig. 28*G*), the chalazal one becomes conspicuously vacuolated and gives rise to the megagameto-

phyte. The outer integument has now reached the length of the inner and begins to exceed it. Centripetal growth of the distal end of the inner integument has reduced the micropylar opening. The chalazal megaspore seems to develop nor-



FIGS. 30-40.—Figs. 30-33, longisections showing development of female gametophyte. Fig. 30, section of micropylar portion of ovule perpendicular to longitudinal axis of funiculus (O, outer; I, inner integument). Fig. 33, longisection of ovule at fertilization, parallel to longitudinal axis of funiculus. Fig. 34, male nuclei beside egg cell. Fig. 35, fertilization. Fig. 36, male nucleus beside fusion nucleus in chalazal region of female gametophyte. Fig. 37, gametophyte after fertilization. Figs. 38-40, first and second divisions in endosperm.

mally into a typical 8-nucleate megagametophyte. From the 2-nucleate stage on, the megagametophyte shows a decided polarity, the nuclei being equally distributed above and below the large central vacuole (figs. 29-31). The polar nuclei were observed in the process of fusion at about the middle of the megagameto-

phyte, after formation of antipodal cells and the egg apparatus. Three antipodal cells are organized (figs. 31, 32, *AP*). They soon show signs of degeneration.

In the later stages of its development the megagametophyte contains abundant starch grains, especially in the micropylar cells and about the fusion nucleus. The nucellar tissue flanking the megagametophyte has begun to disappear (through digestion) by this time. Before fertilization occurs this part of the nucellus has been completely digested, and the sides of the megagametophyte (fig. 33*G*) are in contact with the inner layer of the inner integument.

After degeneration of the antipodals the fusion nucleus comes to lie at the chalazal end of the megagametophyte, which becomes truncate as it extends coincident with digestion of the chalazal nucellus. When ready for fertilization, the megagametophyte contains two synergids and an egg cell in the micropylar end and the fusion nucleus in the opposite end. The outer integument now extends beyond the inner. After the chalazal megaspore has begun to enlarge, or sometimes even before, a palisade of unicellular glandular hairs develops from the superficial layer of the placenta and adjacent regions of the funiculi. These hairs, together with those formed within the ovary near the base of the style, produce a jelly-like, colorless material that fills the ovary before fertilization and persists until after the seed germinates.

#### POLLINATION AND FERTILIZATION

Prior to pollination the spathe remains tightly wrapped about the spadix, one edge overlapping the other. The upper part of the spathe surrounding the staminate flowers becomes marked off from the lower surrounding the pistillate flowers. While the upper two-thirds of the spathe is dark green and tapers gradually toward the tip, the lower part is yellowish to light green and of greatest diameter at the middle. Opening in both regions is preceded by longitudinal extension of first the inner and then the outer margin of the spathe, both margins being thrown into wavelike folds. The lower part of the spathe opens first, but the opening is small and only a few pistillate flowers can be observed from without. Each pistillate flower consists of one to five whitish, fleshy staminodes surrounding a flask-shaped pistil about 3 mm. high with a subglobose, one-celled ovary about 2.5 mm. in diameter; a comparatively thick style 0.8–1.5 mm. long, having a central canal lined with hairs; and an inconspicuous terminal stigma of short unicellular hairs (figs. 14, 15). The upper portion of the spathe opens later. Sometimes it opens completely, freely exposing the tip of the spadix; but it may only loosen a little, without actually exposing the staminate flowers.

The behavior of the spathe and other circumstances make cross-pollination feasible and probable. Insects (Syrphidae, Chloropidae, and others) were seen entering the spathe, frequently laden with *Peltandra* pollen. Eggs of insects are

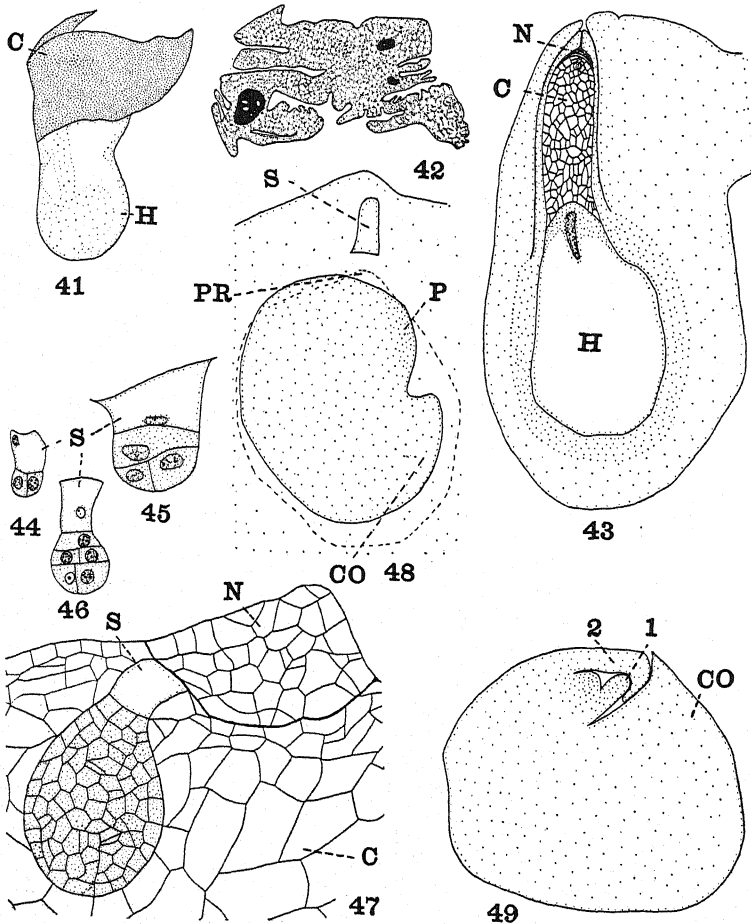
often found on the inner surface of the spathe, and insect larvae develop in and feed on the immature fruiting spadix. In several instances, when only the lower part of the spathe surrounding the pistillate flowers was open, germinated pollen grains were observed on the stigmas of the pistils before there was any sign of dehiscence and liberation of pollen from the loculi of the synandria on the same spadix. Apparently the pistillate flowers can be and are pollinated before the staminate ones on the same spadix liberate pollen. Should cross-pollination of any pistillate flower fail to occur, self-pollination is still possible, if the stigma remains receptive; for large amounts of pollen from the overlying synandria are liberated and reach the lower region of the spadix (by means of insects or force of gravity) after fertilization has occurred in some of the pistillate flowers on the same spadix.

Germinated pollen grains have been observed on the stigma; pollen tubes have been found in the stylar canal, in the jelly-like material within the ovary, entering the micropyle, and traversing the micropylar nucellus. As recorded for several other plants (29), more than one pollen tube may enter the megagametophyte. In one case three pollen tubes entered a single micropyle. In some instances clear areas, like those observed in the pollen tubes, surround the male nuclei in the megagametophyte (fig. 34*M*). One male nucleus (fig. 35*M*), lacking a nucleolus and staining densely, fuses with the egg nucleus; a second fuses with the fusion nucleus (fig. 36*M*). Among aroids double fertilization has also been recorded for *Arum maculatum* (21) and *Arisarum vulgare* (22), and suggested for *Dieffenbachia picta* var. *baraquiniana* (16). The fusion nucleus contains a large vacuolated nucleolus. Upon fusion with a male nucleus, the resulting endosperm nucleus finally comes to possess several smaller nucleoli, and the rather irregular chromatic reticulum is replaced by a more threadlike structure.

#### DEVELOPMENT OF ENDOSPERM

So far as observed, the endosperm nucleus always divides before the oospore nucleus. Usually this nucleus migrates from its chalazal position toward the micropyle (fig. 37), dividing at a position somewhat more than half way (fig. 38). Cytokinesis follows this karyokinesis (fig. 39). In this respect *Peltandra* resembles some aroids (1, 20, 23) but differs from others (15, 23, 26, 27). The micropylar endosperm cell thus formed gives rise to a cellular endosperm (figs. 39, 40, 43, *C*). The chalazal cell (*H*) never divides again and eventually reaches a tremendous size (figs. 41, 43, *H*), 2.4 by 1.6 mm. or more (cf. 26). Its nucleus also fails to divide again, increases greatly in size, and becomes very much lobed (fig. 42). After attaining a large size, the nucleolus fragments and the pieces become highly vacuolated. When endosperm with the chalazal cell still intact was dissected from ovules, mounted in tap water, and observed with the microscope, large coarse strands of cytoplasm were noted traversing the cell vacuole of the giant haustorial

cell. Within these strands the middle regions could be seen in streaming motion. Large haustorial cells, similar in origin and possessing large and intricately lobed nuclei, have been reported for other aroids, but the number found in different species varies from one to eight.



FIGS. 41-49.—Fig. 41, endosperm dissected from ovule, with haustorial cell intact. Fig. 42, section of nucleus of haustorial cell. Fig. 43, longitudinal section of maturing ovule in plane of funiculus. Figs. 44-49, development of embryo (N, nucellar cap).

The second nuclear division in the endosperm, that is, the division of the nucleus of the micropylar daughter cell, is also accompanied by cytokinesis (figs. 39, 40); hence development of the endosperm is cellular. The occurrence of cellular endosperm has been suggested or reported several times for aroids (23, 29, 30). Usually the reports are uncertain or the necessary sequence of stages has not been

obtained. On the other hand, the reports indicate that if the endosperm (especially the micropylar portion) is not cellular from the start, the non-cellular condition is of brief duration (1, 25).

The third division in the endosperm was not observed. Soon walls, perpendicular to those first formed and parallel to the long axis of the ovule, appear in the chalazal region of the cellular endosperm. Eventually the micropylar endosperm cells also undergo divisions in this plane.

In the development of the ovule after fertilization, the haustorial cell at first enlarges more rapidly than the cellular endosperm, especially in a direction perpendicular to the long axis of the ovule. Consequently the products of the megagametophyte (and the entire ovule) assume, as a whole, a pyriform shape, the micropylar end being narrower (fig. 43). In subsequent development of the ovule, the haustorial cell, after attaining relatively great dimensions, ceases to enlarge; it is overtaken and finally surpassed in cross-sectional area, as well as in longitudinal extent, by the cellular endosperm. By the time the embryo sporophyte becomes fairly conspicuous the form of the maturing seed is again pyriform, but this time the chalazal region housing the haustorial cell is the narrower. In the ripe seed the micropylar portion containing the large embryo is by far the more conspicuous; the haustorial cell with its surrounding coat of parent sporophytic tissue becomes a mere withered protuberance of the chalazal region.

#### DEVELOPMENT OF EMBRYO

When the endosperm is in the 2- or 3-cell stage, the oospore nucleus divides and a wall perpendicular to the longitudinal axis of the ovule is formed (fig. 40). The micropylar cell is the larger, and with the possible exception of one preparation was not observed to undergo further nuclear or cellular divisions (figs. 40, 44, 45, 46, 47, 48, 5). It becomes the one-celled suspensor, and although relatively large attains no striking dimensions or differentiation. The smaller distal cell gives rise to the embryo proper, and next divides by a wall perpendicular to the first (fig. 44).

A review of studies on aroids reveals that while the suspensor does not become conspicuous, its origin and the fate of the micropylar cell of the 2-celled proembryo vary in different species. *Pistia* (20) has no suspensor; in *Arum orientale* and *A. maculatum* (20) the suspensor arises from the micropylar cell of the 2-celled proembryo; in *Acorus* (1) and *Antherurus* (17) the micropylar cell divides by a transverse wall; in *Calla* (23) and *Arisaema* (26) cellular suspensors arise from the micropylar cell.

The first and second cell walls to be formed in the embryo are in some cases parallel to each other and perpendicular to the long axis of the ovule, for example, in *Aglaonema* (2) and others. In *Peltandra*, *Arisaema* (26), and *Spathicarpa sagit-*

*taefolia* (3) the second wall in the proembryo is perpendicular to the first and parallel to the long axis of the ovule. The immediately succeeding divisions were not seen, but the 7-cell stage (fig. 45) indicates that the next walls formed are parallel to the first and hence perpendicular to the second. Figure 46 is a longitudinal section of the 10-cell stage.

The embryo proper now proceeds to form a globular to ovoid body (fig. 47). Later a groove appears on one side of the embryo and separates the terminal cotyledon (fig. 48CO) from the lateral plumule primordium (fig. 48P; dotted line is outline of embryo in region of primary root, PR). Except in the region separating the plumule from the cotyledon, the surface of the latter is evenly continuous with the rest of the embryo at this as well as at later stages of embryonic development, for the radicle does not form a distinct protuberance. Initiation of the primary root begins shortly after the cotyledon and plumule primordia have been differentiated.

While the radicle seems to undergo an abortive development, the terminal cotyledon enlarges so rapidly and extensively that the plumule comes to lie roughly at the micropylar end of the embryo as a whole. The growing point of the plumule always points laterally, is usually distinct; and as the cotyledon enlarges, gives rise to the first cauline foliar organ on its micropylar side (fig. 49, 2). The next leaf arises on the chalazal side (fig. 49, 1). At maturity the embryo possesses a relatively large plumule with six or seven cauline leaf rudiments and several rudimentary adventive roots.

In the late summer and fall the lower parts of the spathes around the ripening fruits disintegrate, together with the remaining axial part of the spadix. Since the released fruits are buoyant and the marshes occasionally flooded, they were observed dispersed by flowing water in Maryland.

### Summary

1. The subterranean vertical rhizome of *Peltandra virginica* is built up sympodially from branches arising in the axils of the penultimate leaves of continuation shoots of successively higher rank. Each continuation shoot, as well as the primary axis, terminates in a spadix. A second spadix arises in the axil of the ultimate leaf.
2. Usually vegetative buds, which sometimes result in vegetative multiplication of the plant, arise in the axils of only the antepenultimate leaves.
3. There is a tendency to limit the number of foliage leaves formed on a continuation shoot. The minimum number was three.
4. Leaves and inflorescences appear to be initiated about 20 months before they actually emerge from the terminal bud.

5. Development of the staminate flowers differs from that of the pistillate in that the latter alone show a distinct segmentation of the floral primordia.

6. A "bouquet" stage was found in early meiosis. The haploid number of chromosomes was 56.

7. Periplasmodium formation was found in the microsporangium, and the pollen is 3-nucleate before it is shed. Evidence for the occurrence of cross-pollination was found. "Double" fertilization occurs.

8. The endosperm is cellular from the start; the large haustorial cell in the maturing ovule is the chalazal daughter cell formed upon division of the endosperm cell.

9. The embryo contains a large, well-developed plumule and a small, poorly-developed primary root.

The writer wishes to acknowledge his debt to the late Professor D. S. JOHNSON, under whom this work was undertaken, and to Professor C. O. ROSENDAHL, for helpful criticism of the paper; and also his appreciation for the cooperation of Miss ALMA RUTLEDGE, Mr. MILTON SEIDMAN, and Dr. T. I. EDWARDS. Insects were identified by Dr. ELIZABETH FISHER.

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# COAL FLORA STUDIES: LEPIDODENDRALES<sup>1</sup>

FREDDA D. REED

(WITH THIRTY-FIVE FIGURES)

## Introduction

In the century or more since the appearance of LINDLEY and HUTTON's (10) account of histological studies of *Lepidodendron*, considerable literature has accumulated on the anatomy and morphology of Paleozoic arborescent lycopods. Nevertheless, there is still lacking precise knowledge of specific details of both vegetative and reproductive structures that would serve, not only to give a more complete picture of the group, but also to provide diagnostic criteria for identifying to generic and specific position isolated portions of these fossil plants. The present state of our knowledge does not discriminate between *Lepidodendron* and *Sigillaria* in the case of many of the detached and fragmented lycopodiaceous organs, and for that reason the term Lepidodendrales, an order including all the arborescent lycopods, is used in the above title.

The material here described appeared in coal balls from Harrisburg, Illinois, coal seam no. 5, which is in the Alleghany group of the Upper Pennsylvanian. The coal balls were collected under the auspices of the Illinois State Geological Survey by the late Dr. A. C. NOÉ, who kindly turned them over to me for investigation. The preparations made from them, consisting of thin sections, opaque sections, and celloidin peels, will be deposited with the Noé collection of paleobotanical specimens in the Illinois State Museum at Urbana, Illinois. In these Harrisburg coal balls there are, in addition to the forms to be described here, many plant fragments some of which have already been recorded (8, 12, 13, 14), and there are still other genera awaiting description.

Of the variety of organs, leaves, stems, roots, and sporangia selected for recording and description here, none of them were found in organic association; moreover, none of them were preserved in entirety. They were chosen because all are genera of the Lepidodendrales order and because in every instance certain of the tissues are well preserved.

## Observations

### I. LEAVES

In the following descriptions only the anatomy of the leaf blade is considered. In many instances a leaf could be followed through several serial sections, a dis-

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tance of 1-3 cm. In a few there was some diminution of size toward the apex, but in none of the leaves were both extremities found; hence there was no indication of the length, and—save for the decrease in diameter at the apical region—there were no observations on histological modifications from the base to the apex.

The term *Lepidophyllum* is here applied to those Lepidodendrolean leaves which have a single xylem strand. It was formerly thought that such leaves belonged to *Lepidodendron*, whereas those with a double strand belonged to *Sigillaria*. More recently GRAHAM (5) has observed: "There does not appear to be any fundamental difference between the leaves of *Sigillaria* and *Lepidodendron*. All leaves with a double bundle are attributed to *Sigillaria*, but this character is not constant for that genus."

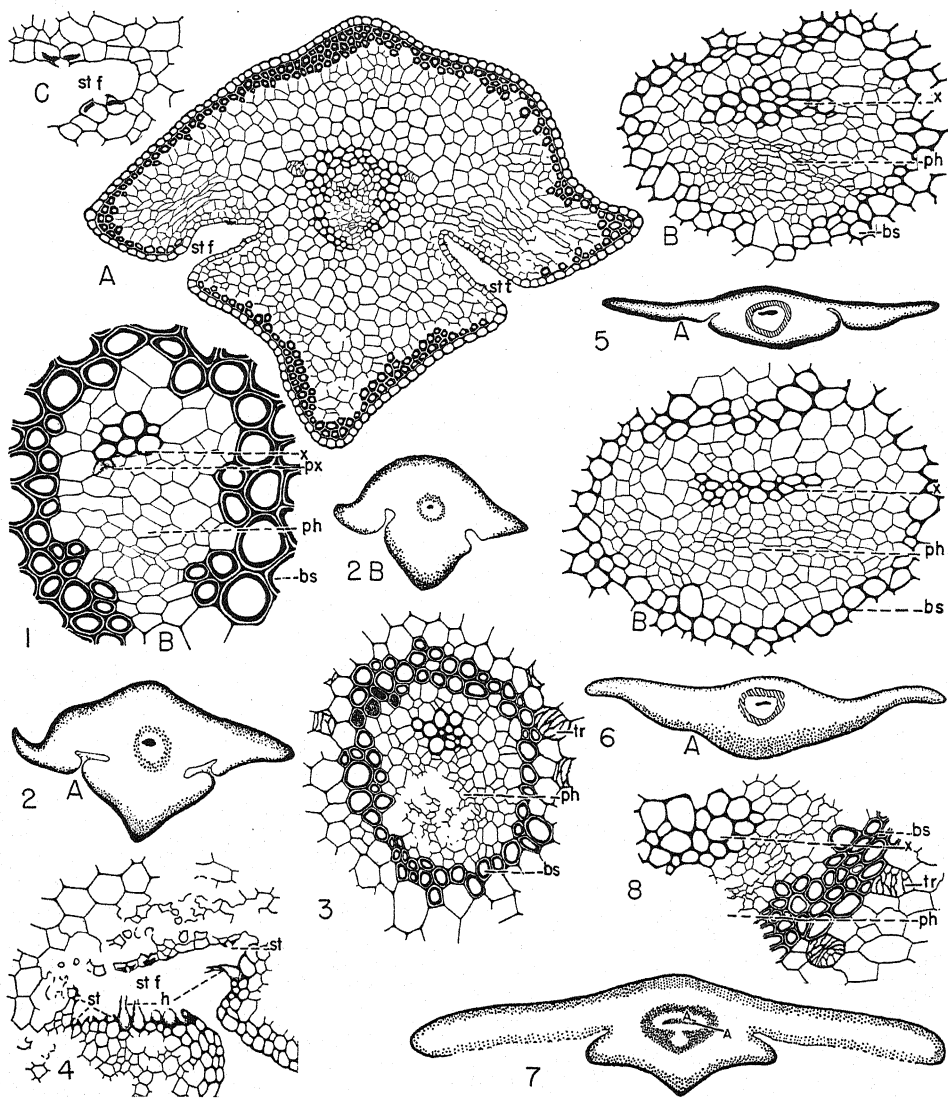
*Lepidophyllum aciculum* sp. nov.

DIAGNOSIS.—Leaves acicular, 0.75 mm. broad by 0.59 mm. thick. Ventral surface convex; dorsal surface convex to angular with two lateral furrows and conspicuous median keel. Vascular strand exarch to mesarch, collateral, surrounded by bundle sheath. Some of cells lateral and exterior to bundle sheath differentiated into transfusion tissue. Mesophyll with no differentiation into palisade cells, mostly compact, but lacunar in region of stomatiferous furrows. Hypodermis one to three cells deep except in region of furrows, where absent. Epidermis of relatively thick-walled cells; stomata confined to furrows.

If there is a lepidodendroid leaf in the Harrisburg material that might be regarded as typical it is this one, for almost every coal ball containing leaves has some fragments of it. The leaf is fine and needle-like; in its known dimensions it is comparable with the leaves of *Pinus strobus*.

The single xylem strand consists of six to eight elements with scalariform markings. The protoxylem was not definitely determined, but on the abaxial side there is some irregularity of cells, either from incomplete preservation or from some disintegration while the plant was living, and that irregularity may represent the position of the protoxylem (fig. 1 Bpx). The vascular bundles of *Lepidophyllum* have been interpreted as concentric (5), but in this leaf, as in the succeeding ones, below the xylem there is a group of thin-walled cells, irregular in size, with the aspect of phloem tissue (fig. 1 A, Bph). Surrounding the xylem and phloem is a layer, one or two cells deep, of large thin-walled parenchyma, and this layer in turn is succeeded by the thick-walled fibrous cells of the bundle sheath, three to four cells deep. In the leaf illustrated the bundle sheath is not complete on the abaxial side (fig. 1 B). For the most part the mesophyll is of undifferentiated thin-walled cells with few and small intercellular spaces, yet in the marginal region the cells are slightly elongated and the intercellular spaces are more conspicuous.

The hypodermis, of small, regular, thick-walled, and isodiametric cells, sur-



FIGS. 1-8.—*Lepidophyllum*: Fig. 1, *L. aciculum*. A, transection of leaf: *st f*, stomatal furrow. B, vascular strand of A: *x*, xylem; *px*, protoxylem; *ph*, phloem; *bs*, bundle sheath. C, detail of stomatal furrow at left of A. Noé coll. H 236 1. Figs. 2-4, *L. trichosulcata*. Fig. 2, diagrams of transections: A, median section; B, same leaf from apical region. Noé coll. H 227 A 22. Fig. 3, detail of vascular strand of 2 A: *tr*, transfusion tissue. Fig. 4, detail of stomatal furrow at right of 2 A: *st*, stoma; *h*, epidermal hair. Fig. 5, *L. alatum*: A, diagram of transection; B, detail of vascular strand. Noé coll. H 236 13. Fig. 6, *L. latifolium*: A, diagram of transection; B, detail of vascular strand. Figs. 7, 8, *L. thomasi*. Noé coll. H 236 12. Fig. 7, diagram of transection. Fig. 8, detail from A—A of fig. 7.

rounds the mesophyll save for the region of the furrows, but it is thickest above and below the vascular strand and at the margins (fig. 1 A). The epidermis is likewise of small regular cells, but the walls are not so thick as are those of the hypodermis. About the furrows the walls are thinner, and here are found the stomata (fig. 1 C). These were apparently crowded, for often there is only one epidermal cell separating two guard cells.

*Lepidophyllum trichosulcata* sp. nov.

DIAGNOSIS.—Leaves about twice as wide as thick, 1.8 mm. by 0.94 mm., tapering to 1 mm. by 0.7 mm. Ventral surface convex; dorsal surface angular with conspicuous median keel and two lateral stomatiferous furrows. Vascular strand exarch to mesarch, collateral, and inclosed by bundle sheath of thick-walled fibrous cells. Transfusion elements in tissue surrounding bundle sheath. Mesophyll thin-walled, irregular cells but not differentiated into palisade tissue; larger lacunar areas in region of stomatiferous furrows. Hypodermis two to four cells deep except around furrows, where absent. Epidermis of relatively small and regular cells, except in furrows, where their walls are thinner and some cells are differentiated into guard cells, others into hairs which extend into the furrows.

While the general aspect of *Lepidophyllum trichosulcata* differs markedly from that of *L. aciculium*, yet a comparison of the diagnoses reveals but two essential differences: (1) the difference in size and shape, which would of itself not be considered a specific character but in combination with structural differences does have some diagnostic value; and (2) the cellular organization about the lateral furrows. *L. trichosulcata* in its dimensions of width and thickness measures more than twice that of *L. aciculium*. The leaf was observed in several sections, in the last of which is the apex; following it through it is seen to taper gradually to dimensions of about half that of the maximum size and to vary somewhat in the shape of the transverse section (fig. 2 A, B).

The xylem in section (fig. 3) is a circular to elliptical group of twelve to twenty tracheids, the number diminishing toward the apex. In this bundle, as in so many of the specimens of *Lepidophyllum*, the tissue below the xylem is incompletely preserved. The remaining cells are somewhat irregular in shape and are thin walled. They were not observed in longitudinal section, and sieve tubes have not been demonstrated; but—as in the former leaf—it is this tissue that is interpreted as that of phloem. Surrounding the xylem and phloem are two to three layers of parenchymatous cells and then the sheath of thick-walled fibrous cells (fig. 3). Exterior to the bundle sheath are occasional transfusion elements with reticulate to scalariform thickenings (fig. 3|).

Neither the cells of the mesophyll nor those of the hypodermis show much differentiation, nor are the two regions sharply delimited from each other. The cells of the hypodermis are more regular but their walls are only slightly thicker.

The most distinguishing feature of this leaf is the cellular organization about the stomatiferous furrows. Many of the epidermal cells in the vicinity of the opening are elongated into hairs which extend into the chamber (fig. 4). Distributed all around the remaining part of the furrow are the stomata, numerous and crowded—eight are indicated at the level shown in figure 4. The hairs, fine and delicate, measuring about  $6\ \mu$  in diameter, are demonstrable only under oil immersion lens. In transverse section the stomatal furrows or grooves bear some resemblance to the stomatal chambers of *Nerium*, except that in *L. trichosulcata* the hairs are assembled near the opening of the groove as if to guard the entrance and are not interspersed irregularly among the stomata as are those of *Nerium*. The whole mechanism would appear to be very effective in guarding against excessive water loss.

*Lepidophyllum alatum* sp. nov.

DIAGNOSIS.—Leaf more than five times as wide as thick, 2.25 mm. wide by 0.42 mm. thick. Ventral surfaces slightly convex; dorsal surface convex with two lateral furrows. Vascular strand probably exarch, collateral, inclosed by bundle sheath. Xylem strand in transverse section of about twenty elements arranged in horizontally elongated group. Mesophyll undifferentiated, surrounded by hypodermis, except in region of lateral furrows. Stomata not observed, but thin-walled epidermis and absence of hypodermis about the lateral furrows would indicate that they were confined to that region.

This leaf (fig. 5 A, B) is broader and thinner than that of the preceding two species. In its dimensions of breadth and thickness it is comparable with *Taxus canadensis*; also, these dimensions are in about the same proportions to *L. aciculum* and *L. trichosulcata* as are the dimensions of *T. canadensis* to *Pinus strobus* and *P. rigida*, respectively.

Except for the difference in size and shape and the horizontal arrangement of the xylem elements, this leaf is not especially noteworthy.

*Lepidophyllum latifolium* Graham

The type specimen of *L. latifolium*, described by GRAHAM (5), came from the Lower Coal Measures of Great Britain and is in the Binney collection, Sedgewick Museum, Cambridge.

The size of the Harrisburg leaf, 2.4 mm. wide by 0.5 mm. thick, is somewhat smaller than that of the type specimen, but it lies within the limits of variability as recorded for the species. In both leaves, this (fig. 6 A, B) and the type specimen, the xylem in section is in a horizontally elongated band. While there are more elements in this leaf (twenty xylem elements shown in fig. 6 B), yet the variation in number is not greater than might be found in one strand in its course from base to apex. The disposition of the hypodermis of the two leaves is the same,

in that it is absent, or one cell thick, on the adaxial side of the leaf and from five to six cells thick on the lower side. In other details there is so little variation from the type specimen that there seems no valid reason for setting the Harrisburg leaf apart into a new species.

*Lepidophyllum thomasi* Graham

The species *L. thomasi* was established by GRAHAM (5) for the reception of some lycopodiaceous leaves which were also from the Lower Coal Measures of Great Britain. Of all the specimens of *L. thomasi* in the Harrisburg collection, there is none entirely comformable with the type specimen; however, the kind as well as the degree of variation appears to be individual rather than specific.

The leaf of *L. thomasi* is the largest of the lepidodendroid leaves discovered in this particular collection of coal balls. The length is certainly several centimeters, and some of the transverse sections measure up to 6 mm. in width and 2.5 mm. deep. Usually they are about four times as wide as deep.

There are two distinguishing features that make *L. thomasi* recognizable, even though the leaves may be folded or crushed, partially preserved or fragmented; they are the very characteristic hypodermis and the pattern of the vascular strand. The hypodermis is a sharply defined and massive layer six to ten cells deep. It is thickest above and below the midrib and less well developed on the lower side of the lamina (fig. 7). The cells in transverse section are isodiametric and regular, thick walled, and arranged in radial rows; in tangential section all, except those of the lower side of the lamina, are elongated.

In keeping with the superficial dimensions of this leaf, the vascular strand is larger and composed of more elements than are the strands of the preceding species. The xylem elements, numbering up to fifty or more in the larger bundles, are arranged in a horizontally elongated group as in *L. alatum* and *L. latifolium*. The position of the protoxylem is uncertain, but there are smaller elements at either end of the metaxylem which may be those of the protoxylem (figs. 7, 8). The xylem is surrounded by a tissue of thin-walled irregular cells which is considerably more extensive on the abaxial side (figs. 7, 8); it is this tissue that is labeled phloem.

The bundle sheath and inclosing elements distinguish the vascular strand. Laterally and adaxially the sheath is an elliptical mass of thick-walled fibrous cells (fig. 7), appearing much as in other species of *Lepidophyllum*. On the abaxial side, however, it extends downward into a U-shaped pattern. The U is filled with thin-walled parenchymatous tissue, tissue that may be secretory in nature, as has been described in other instances (4, 1, 20), or may be—as sometimes appears—simply a continuation or extension of the phloem. In addition to the two species of leaves described by GRAHAM (5), *L. thomasi* and *L. papillonaceum*, this same type of

bundle sheath, although not always interpreted as such, has been found in the leaf traces of *Lepidodendron harcourtii* (1), in the leaf traces of *Lepidophlois fuliginosus* (20), and in the cone scale of *Lepidostrobus* (4). Also CHRYSLER has figured a leaf labeled *Sigillaria*, from the Foot Mine, Shore, Littleborough, Lancashire, which shows the same organization of thick-walled elements about the conducting strand (4).

### *Sigillariopsis* Renault

The genus *Sigillariopsis* was established in 1879 by RENAULT (15) for a small stem with leaves attached from the Permian of Autun. Both stem and leaf were essentially lycopodiaceous, the leaf differing from previously described genera in the possession of two parallel xylem strands. In 1904 SCOTT (18) applied the term *Sigillariopsis* to some lycopodiaceous leaves with two xylem strands from the Lower Coal Measures of Lancashire; these leaves were described under the specific name of *S. sulcata*. Subsequently the genus *Sigillariopsis* has become a repository for leaves with a double xylem strand (2, 7, 5), but in only one instance has a specimen been found in close association with *Sigillaria* (2).

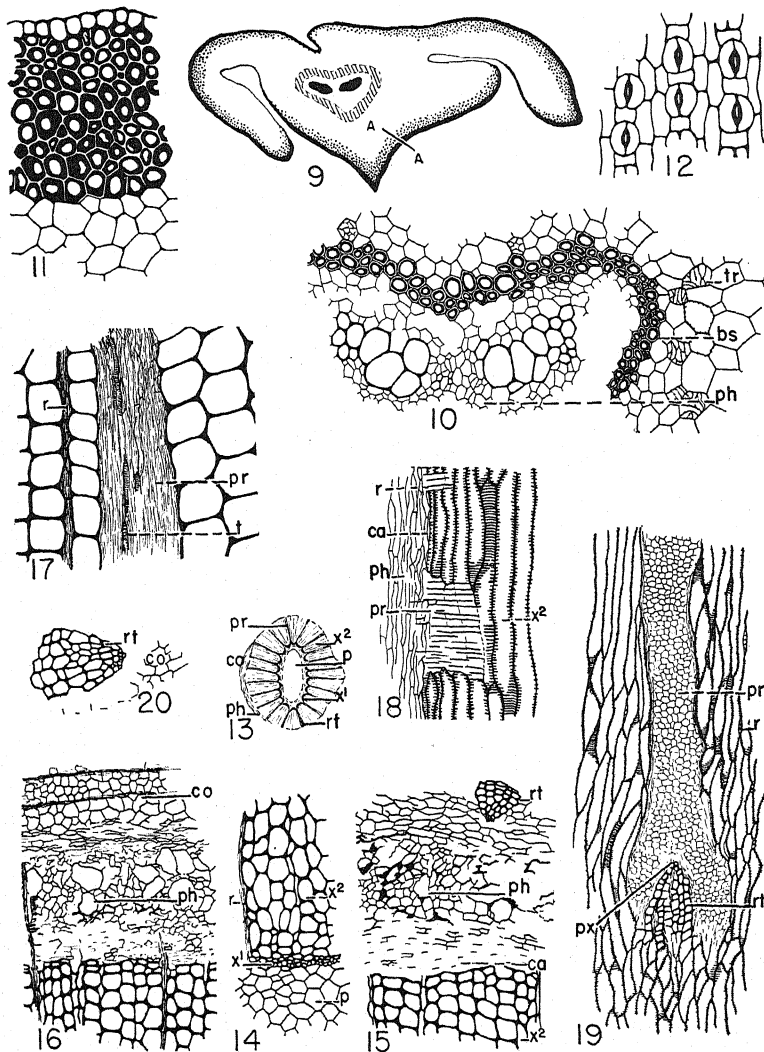
### *Sigillariopsis cordata* sp. nov.

DIAGNOSIS.—Leaves broad with thin laminal margins, 3 mm. wide by 1 mm. thick at midrib and 0.25 mm. thick in marginal region. Ventral surface slightly convex, with shallow median depression; dorsal surface with prominent median keel and lateral stomatiferous furrows. Central vascular region has two parallel strands of xylem with smaller elements at outer and upper end of each strand. Collateral bundle surrounded by bundle sheath two to three cells deep; bundle sheath in transverse section broadly heart-shaped. Some cells exterior to bundle sheath differentiated into transfusion elements, which are more numerous laterally than above or below bundle sheath. Mesophyll not differentiated into palisade cells, lacunar in region of furrows. Stomata, confined to furrows, in regular linear rows.

*Sigillariopsis cordata* has been found in very few of the coal balls investigated, but when it does occur it is abundantly represented and is remarkably well preserved. Usually not all the tissues are equally well preserved in one leaf nor at any one level, but in the assemblage of leaves it is possible to work out the structure in considerable detail. Figure 9 shows the position and relation of several tissues in transverse section. Save for the bundle sheath, it appears extraordinarily like *S. halifaxensis* Graham, from the Halifax Hard Beds, Lower Coal Measures, Great Britain (5). The cellular detail, however, reveals structural features not in accord with the description of *S. halifaxensis*.

The xylem is organized in two median parallel strands, each composed of nine to





FIGS. 9-20.—Figs. 9-12, *Sigillariopsis cordata*: Fig. 9, diagram of transaction. Noé coll. H 2. Fig. 10, detail of portion of vascular region showing two xylem strands. Fig. 11, epidermis and hypodermis from A—A of fig. 9. Fig. 12, surface view of epidermis from stomatal furrow showing stomata. Figs. 13-20, *Stigmaria ficoides*: Fig. 13, diagram of transaction: *p*, pith; *x*<sup>1</sup>, primary wood; *x*<sup>2</sup>, secondary wood; *pr*, principal ray; *rt*, root trace; *ph*, phloem; *co*, cortex. Noé coll. H 204. Figs. 14-17, portions from different regions of transaction: *ca*, cambium; *r*, wood ray. Fig. 18, from radial section. Fig. 19, from tangential section showing portion of principal ray and rootlet trace. Fig. 20, rootlet trace with secondary wood.

fifteen elements, with smaller elements at the outer and upper ends (fig. 10). The surrounding parenchyma is for the most part irregularly and poorly preserved, but that below the xylem is more extensively developed, is irregular in size and shape of cells, and appears to be the region of the phloem. The bundle sheath is a broadly heart-shaped band of thick-walled cells, one to three cells deep (figs. 9, 10). Exterior to the bundle sheath are the transfusion elements with delicate reticulated to scalariform markings on their walls (fig. 10tr). The transfusion tissue does not form a complete zone about the vascular strand; but as in *Lepidophyllum*, there are more thickened elements at either side of the vascular strand than above or below it.

The hypodermis is a striking feature of this leaf. It is thickest about the abaxial keel, thinnest in the region of the adaxial median groove, and absent about the stomatiferous furrows. The hypodermis is composed of tangentially elongated cells which are roughly isodiametric in transverse section (fig. 11). The lumina of the cells are often filled with some dark material, and this in combination with the extremely thick walls renders the hypodermal layer almost opaque.

Stomata were found only about the lateral abaxial furrows. A surface view of the epidermis about the furrows shows a regular and unique stomatal pattern. The cells are arranged in longitudinal rows; the stomatal rows, consisting of guard cells alternating with short and broad epidermal cells, are separated by two rows of long and narrow epidermal cells (fig. 12).

## II. STIGMARIA AND STIGMARIAN ROOTLETS

### *Stigmaria ficoides* Br.

In Carboniferous floras there is probably no genus more widely distributed or more abundantly represented than is *Stigmaria* and its appendages, stigmarian rootlets. Fossils of *Stigmaria* are found with structure preserved in coal balls, and they are found as casts and impressions in the shales interbedded with the coal. Yet for the number of specimens and the variety of methods of preservation, there is great uniformity in the superficial features as well as in the anatomical structure. Comparatively few species, not more than a dozen, based upon external characters of form and size have been recorded, and only three or four species established upon knowledge of internal anatomical structure. Of the specimens in the latter category, by far the greater number fall into the species *S. ficoides*. When the variation in the superficial characters is considered, however, and also the fact that *Stigmaria* is the basal or rhizomatous portion of the many species—more than 150—of *Lepidodendron*, *Sigillaria*, and *Bothrodendron*, it would seem that among the many specimens there should be some expression of specific difference in anatomical detail thus far not discovered. Even so, in the following description

there seems no justification for regarding the specimen as other than *S. ficoides*, as it is diagnosed.

*Stigmaria ficoides* has been described from American coal fields (6), but the writer has not given the locality nor the horizon from whence it came other than the Pennsylvanian of Illinois. While describing his specimen as *S. ficoides*, HOSKINS (6) makes this reservation: "recognizing the probability that the rather large variation of forms now included in this species may eventually be separated specifically."

The fragment here considered is a piece of wood about 15 cm. long by 2.5-3 cm. in diameter. On one side the phloem and a portion of the cortex is intact. The fragment was so largely composed of pyrite that the thin sections were opaque, but the structural features were discernible in reflected light. A diagram of the transverse section shows the extent of the preservation (fig. 13). The central cavity is encircled by a narrow zone of pith, beyond which is the primary wood, limited in amount and with the smallest elements toward the center (fig. 14 $px$ ). Secondary wood makes up most of the fragment. The tracheids with scalariform markings on both radial and tangential walls are disposed in regular radial rows (figs. 14, 15, 18, 19), with one to seven rows separated by uniseriate rays one to six cells deep (figs. 15, 16). The wood, both primary and secondary, in transverse section has the appearance of organization into discrete wedge-shaped bundles separated by broad parenchymatous rays which SCOTT (19) has termed the "principal rays." In tangential section the principal rays are seen as lenticular masses in spiral succession. These masses of tissue are quite ten times as long as wide, which is in contrast with previously described specimens where the length is scarcely twice that of the width (6, 19). The thin-walled, radially elongated cells of the ray (fig. 17) are subtended by the spiral and scalariform tracheids of the rootlet trace (fig. 19 $rt$ ). In the lower part of the rootlet trace the tracheids are continuous with and merge into those of the vascular cylinder.

This specimen, unlike most, has retained some of the softer and more fragile tissues beyond the secondary wood (figs. 13, 15, 16, 18). The preservation is poor and the tissues incomplete; nevertheless there is enough to show a cambial region of relatively small, regularly arranged, thin-walled cells, succeeded by phloem of larger and irregular cells. The phloem in turn is bounded by the inner cortex, a region (if one may rely on the integrity of the preservation) composed of alternating layers of tangentially elongated cells and isodiametric cells of larger caliber (fig. 16). The inner cortex is incomplete and there is no preservation beyond it.

STIGMARIAN ROOTLETS.—Almost everyone who has worked with coal ball material has commented upon the abundance and frequency of stigmarian rootlets. They are found not only intermingled with other vegetable debris, but they penetrate tissues as the pith region of stems of any and all genera of contemporary plants.

Rootlet traces in the stele and inner cortex have secondary wood (figs. 15, 20). Occasionally a free rootlet is encountered with secondary wood, but most of them have primary wood only. Usually only the thick-walled cells, which are those of the outer cortex and xylem, are preserved; but some are found with sufficient preservation to demonstrate a central position of the triangular monarch stele, while others demonstrate just as clearly an eccentric position (fig. 21 A, B).

### III. REPRODUCTIVE STRUCTURES

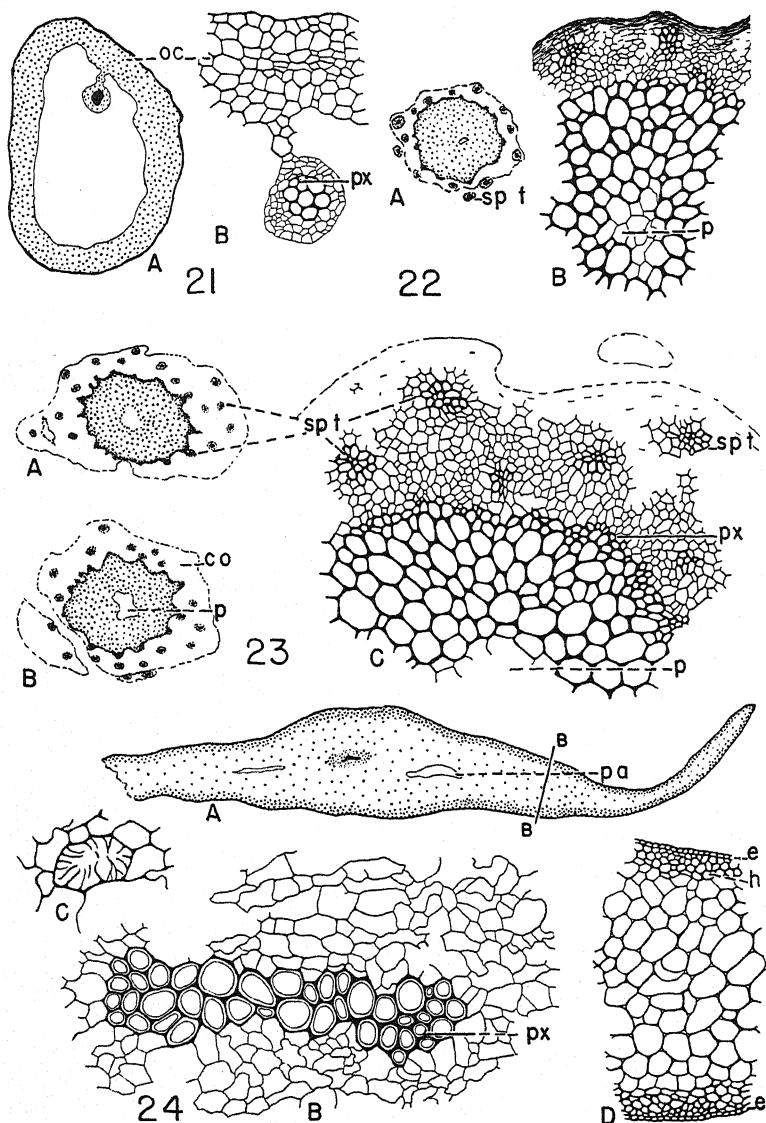
#### *Lepidostrobus*, axis of strobilus

There were two fragments of cone axes in coal ball H 227, and while the two may have originated from the same strobilus, yet the variation in size and the relative proportion of the several tissues indicate an origin from separate strobili. The smaller axis measures about 1.7 mm. in diameter and the larger about 2 mm. The larger fragment is also longer and shows some diminution in size at successive levels, which is in accord with a previously described *Lepidostrobus* axis (3).

In both specimens there is a central pith. It is limited to some fifteen thin-walled parenchymatous cells in the smaller axis (fig. 22 B), while in the larger it is more extensive but the only parenchyma cells remaining are the few disintegrating ones about the periphery of the central cavity (fig. 23 C). Surrounding the pith is the relatively broad zone of primary wood organized with the larger elements toward the center, and the smaller, protoxylem, arranged in fifteen to eighteen peripheral clusters projecting from the surface of the woody cylinder (figs. 22 A; 23 A, B). The xylem is surrounded by a zone of thinner-walled tissue, the diameter of which approximates or exceeds that of the wood (figs. 22 B; 23 C). In this region it is not possible to delimit phloem from cortex, but it is assumed that the innermost cells are those of the phloem while the outermost are doubtless cortical. Imbedded in this tissue are the sporophyll traces (figs. 22 B; 23 C, *sp t*). At any one level these are seen in different stages in their passage from the protoxylem points, where they have their origin, through the cortex. Such a distribution of traces indicates not only spiral succession, but also that they pursued an oblique course. The sporophyll trace bundles are concentric.

#### *Lepidostrobus*, cone scale

Perhaps the most complete description of a *Lepidostrobus* cone scale is CHRYSLER's (4), based upon material from the Lower Coal Measures of Great Britain; but CHRYSLER neither applies a specific name nor does he feel convinced that the scale belongs to the genus *Lepidodendron*, as the name *Lepidostrobus* implies. Although the cone scales here considered differ from those described by CHRYSLER in certain specific details, yet for these too a species name is withheld, inasmuch as it seems possible and probable that the scale or sporophyll will be found attached to



FIGS. 21-24.—Fig. 21, *Stigmara ficoides*, rootlet. A, diagram showing excentric position of stele: *oc*, outer cortex. B, detail of stele and portion of outer cortex: *px*, protoxylem. Figs. 22, 23, *Lepidostrobos*, axis. Fig. 22 A, diagram of transection: *sp t*, sporophyll trace. B, portion from pith to periphery of 22 A. Fig. 23, A and B the same axis at different levels. C, detail from pith to periphery of A. Fig. 24, *Lepidostrobos*, cone scale or sporophyll. A, diagram of transection: *pa*, parichnos. B, vascular strand of A. C, transfusion element from A. D, portion of transection from B—B of A: *e*, epidermis; *h*, hypodermis. Noé coll. H 227.

a sporangium, and if so, a specific name now applied might be rendered superfluous.

The shape of the scale in section and the distribution of the tissues are shown in figure 24. The scale is not only essentially foliaceous, but—aside from the size—bears a strong resemblance to *Lepidophyllum*. The longest fragment measured was about 6 cm. This does not represent the entire length, for the basal end was not preserved. The lower end measures 17 mm. wide by 2 mm. thick. From these dimensions it tapers gradually to an acuminate tip, but still remains relatively thick, being about 0.7 mm. thick where it has diminished to 3 mm. in width.

The xylem of the central vascular strand consists of some forty elements arranged in a horizontally elongated band, with the smaller elements at either end (fig. 24 B). There is some indication that phloem surrounds the xylem, but—as in *Lepidophyllum*—there is a greater display of irregular thin-walled cells on the abaxial side. Unlike *Lepidophyllum* or *Sigillariopsis*, there is no encircling sheath of fibrous cells; but like those foliar organs, there are some transfusion elements lateral and adjacent to the vascular strand (fig. 24 C). At either side of the vascular strand, and separated from it by the width of the strand itself, are tangentially elongated lacunar areas comparable in position and appearance with the parichnos of the leaf bases of *Lepidodendron* and so labeled here (fig. 24 A). In this cone scale the two parichnos strands parallel the vascular strand to within 1 cm. of the tip. The mesophyll is differentiated into an inner zone of large cells (roughly circular in transverse section) and small intercellular spaces and an outer more compact zone, four to five cells deep (fig. 24 D). The cells of the outer zone are of smaller caliber and have slightly thicker walls. The epidermis is a uniform layer of small, relatively thick-walled cells. No stomata were observed.

In its known dimensions and general appearance the cone scales here recorded resemble those described by CHRYSLER (4). The variation is in the organization of the conducting elements and adjacent tissue. The bundle of the latter, with its surrounding cells which form a U-shaped pattern on the abaxial side, is reminiscent of the vascular strand of *Lepidophyllum thomasi*, while the bundle of the former more nearly resembles that of *L. alatum* or *L. latifolium*.

Although these cone scales are five to ten times as wide as any of the species of *Lepidophyllum* just described, yet it is not size that precludes their inclusion among foliar organs of *Lepidodendron*, for many of the species of *Lepidophyllum*, based upon impressions, are as large or larger (9, 11). It is rather their structure, in harmony with that of the pedicel subtending the sporangium, that establishes these organs as the distal laminal portion of the sporophyll of *Lepidostrobus*; and while organic connection is lacking, yet the evidence—that of association and histological similarity—indicates relationship with *Lepidocarpon*.

*Lepidostrobus*, microsporangium and spores

This description is based on a single and isolated specimen consisting of the pedicel of a sporophyll bearing on its adaxial face a sporangium in which are tetrads of small spores. Despite its being a sole specimen, it possesses several features that are unique, distinctive, and specific.

The pedicel is incompletely preserved, but its general shape is outlined in figure 25 A-G (a series of diagrams of tangential sections from the proximal to the distal region of the sporangium). Proximally the pedicel is narrow, with a sharp abaxial keel, but it becomes progressively broader and flatter, with the laminal margins curving about the base of the sporangium. The xylem of the single, median, concentric vascular strand is composed of some thirty elements, arranged in a horizontally elongated to elliptical mass (fig. 26 B). As in the *Lepidostrobus* cone scale, there is no sheath of fibrous cells about the vascular strand.

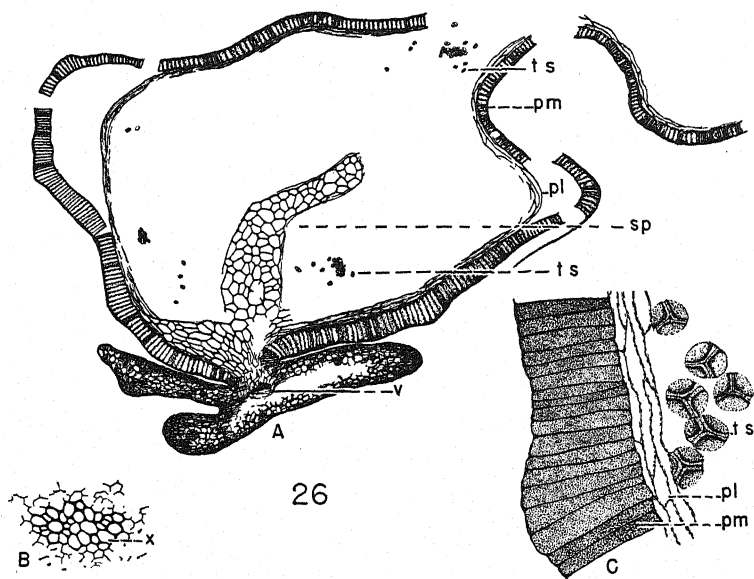
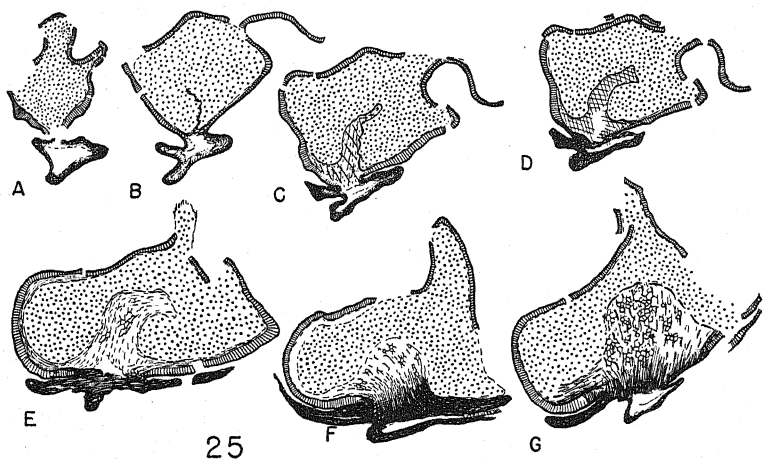
The dimensions of the sporangium, 10 by 2 by 2 mm., are only approximate, for it has been partially crushed; moreover, a small but indeterminable portion of the radial dimension—radial with respect to the axis of the strobilus—was lost in sectioning. The wall of the sporangium is composed of two layers: an outer unicellular and conspicuous layer of radially elongated, thick-walled cells and an inner layer, three cells deep, of thin-walled cells, elongated at right angles to those of the former layer (fig. 26 C). There were no recognizable tapetal cells.

A specific feature of this sporangium is the structure and extensive development of the subarchesporial pad. In the tangential section nearest the axis of the cone there is no indication of sterile tissue within the sporangium (fig. 25 A). In the next section there is a thin band of sterile tissue extending from the base of the sporangium into the sporangial cavity for a distance of one-third the diameter of the sporangium (fig. 25 B). In succeeding tangential sections the tissue becomes broader and thicker, forming in the distal region a massive dome of sterile tissue (fig. 25 G). The cells composing the subarchesporial pad are relatively thin-walled, isodiametric, and large, with an average diameter of 60–70  $\mu$ .

The sporangial cavity is filled with innumerable immature spores which are not separated from one another but are in the tetrahedral formation as they emerged from the spore mother cell. The tetrad is about 30  $\mu$  in diameter. At this stage the spores are free from superficial ornamentation (fig. 26 C).

*Lepidostrobus*, megaspores (*Triletes* Reinsch emend, Schopf)

It is rare that a sectioned coal ball will not yield isolated megaspores; among the megaspores there is considerable variation in size and configuration of spore coat, but thus far they have been consistently alike in the amount of preservation, being represented in almost every instance by the thick impervious spore coat only. When an occasional megaspore has been found with some content (fig. 29), it has



FIGS. 25, 26.—*Lepidostrobus*, microsporangium. Fig. 25, diagrams of tangential sections from proximal to distal region of sporangium. Noé coll. H 227. Fig. 26 A, tangential section of microsporangium: *pm*, prismatic layer of wall of sporangium; *pl*, parietal layers of same; *ts*, tetrads of spores; *sp*, subarchesporial pad; *v*, vascular strand. B, xylem strand of A. C, detail of wall layers and tetrads of spores from A.



been valueless in making further contribution to the development and organization of the megagametophyte, for the content is noncellular and so indistinct that it is impossible to tell whether it is an integral part of the megaspore or some intruded material. The few megaspores selected for description here fall within the dimensions and proportions of *Triletes*, an established genus for megaspores of Paleozoic lycopods (16). While they demonstrate variation in size, shape, ornamentation, and thickness of spore coat to the extent of specific differences, yet because they are not known in all their dimensions and in their surface detail, they have not been assigned species names; instead they are recorded as "types."

Type I.—This is not only the largest of the megaspores, excepting that of *Lepidocarpon*, but it also has the thickest spore coat. It is an almost spherical body with an average inside diameter of about  $850\ \mu$ . The spore coat is about  $85\ \mu$  thick. Both megaspores of figure 27 give evidence of a pronounced equatorial flange and apical suture; otherwise the surface is smooth. The only indication of multiple layers of the spore coat is seen in figure 27 A, where a layer (designated the endospore) is partially separated and infolded.

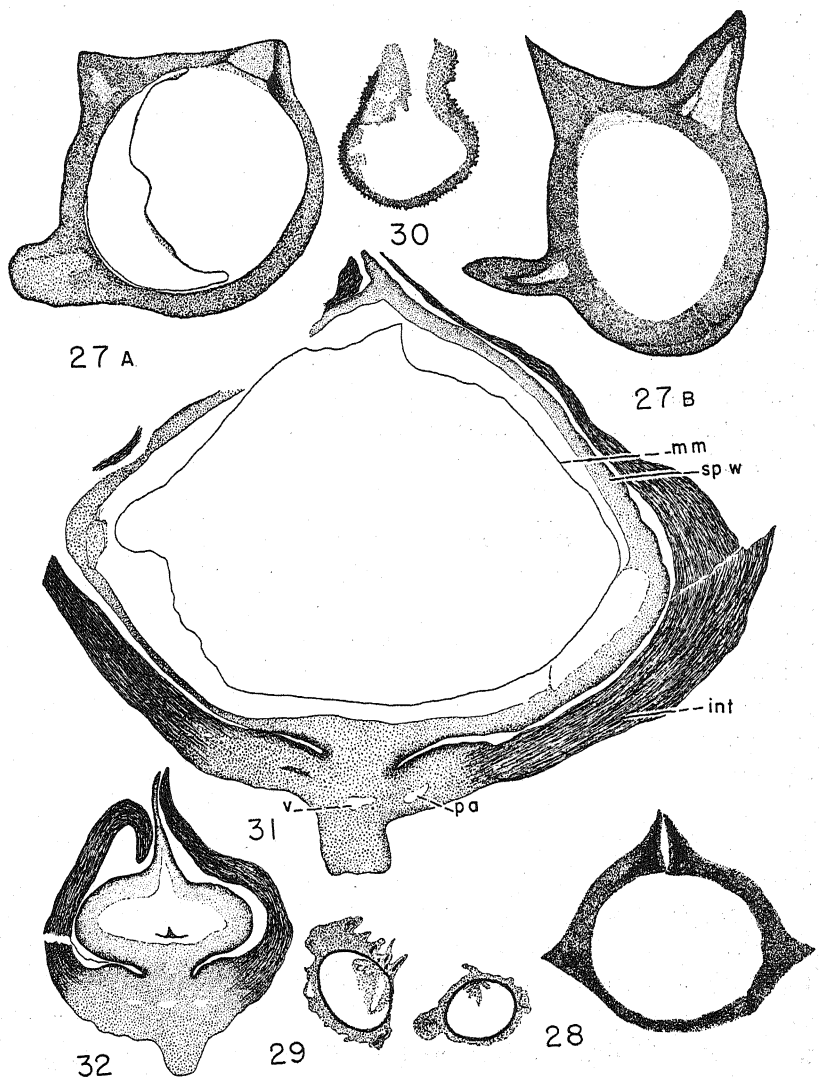
Type II.—The spore body is slightly broader than long, with an equatorial diameter of  $607\ \mu$  and an axial diameter of  $514\ \mu$ . The spore coat averages about  $60\ \mu$  thick (fig. 28). The spore coat is not so thick as is that of the preceding type, and both apical suture and equatorial flange are much less pronounced. There is no observable differentiation into multiple layers.

Type III.—The spore body has an equatorial diameter of  $480\ \mu$  and an axial diameter of  $360\ \mu$ ; these are inside dimensions, exclusive of the spore coat. The spore coat is differentiated into two distinguishable layers: an inner layer, that appears to be homologous with the endospore of the spores of modern pteridophytes, and averages about  $20\ \mu$  thick; an outer layer, perispore, that is wrinkled and folded, and variable in thickness from places so thin as to be scarcely visible to  $150\ \mu$ . Within the spore is some vague and indistinct content, as already mentioned (fig. 29).

Type IV.—This spore is longer than broad, with an axial diameter of  $537\ \mu$  and an equatorial diameter of  $450\ \mu$ . The spore coat averages about  $30\ \mu$  thick. There is no equatorial flange nor uneven and thick perispore, as in the preceding types, instead—in so far as may be judged from a section of the spore—the surface was uniformly and rather finely reticulated (fig. 30).

*Lepidocarpon lomaxi* Scott (17)

In an earlier paper (12) some young unintegumented specimens of *Lepidocarpon* were described. Subsequent investigation has revealed other specimens of such forms as well as those with integuments fully developed. Some of the specimens in the former category possess certain details of preservation that were lacking in



FIGS. 27-32.—Figs. 27-30, *Triletes*: Fig. 27, A and B, Type I. Noé coll. H 239. Fig. 28, Type II. Noé coll. H 227. Fig. 29, Type III. Noé coll. H 216. Fig. 30, Type IV. Noé coll. H. 236. Figs. 31, 32, *Lepidocarpum lomaxi*. Fig. 31, diagram of median tangential section: *mm*, megaspore membrane; *sp w*, wall of sporangium; *int*, integument; *v*, region of vascular strand; *pa*, parichnos. Fig. 32, another integumented specimen of *Lepidocarpum* drawn to same scale as that of fig. 31.

the previous description and so provide supplementary information; whereas in the latter category are specimens which not only show the structure of the mature sporangium and its inclosing integument, but also serve to sustain if not to substantiate some postulated theories with regard to the nutrition of the megagametophyte.

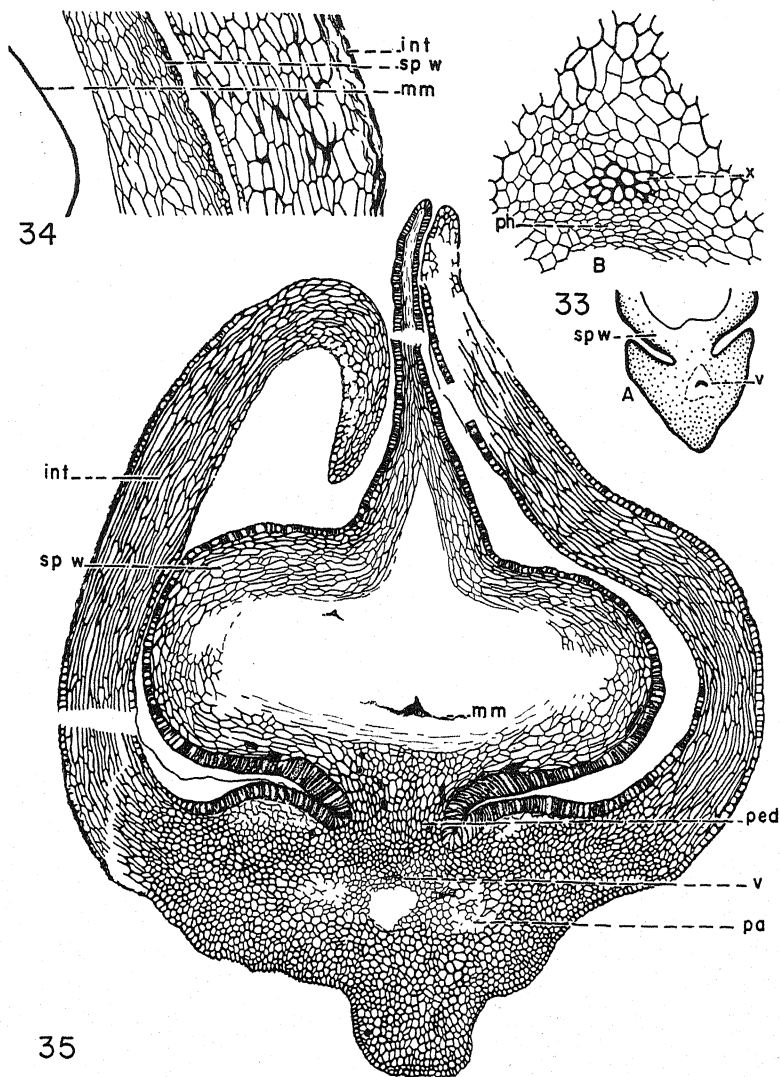
In the previous account some of the specimens indicated the position of the vascular strand of the pedicel, but in none of them was it sufficiently preserved to show the organization of the vascular elements. Figure 33 A is a diagram of the basal portion of tangential section of *Lepidocarpon* showing the position of the vascular strand and its relation to the tissues of the pedicel and sporangium, and a detail from A (fig. 33 B) shows the cellular organization of the xylem and phloem. The vascular strand resembles that of some of the species of *Lepidophyllum*, particularly *L. alatum* and *L. latifolium*; but—as in the cone scale and sporophyll pedicel of *Lepidostrobus*—it is not inclosed by a sheath of thick-walled fibrous cells.

Mature specimens of *Lepidocarpon* are recognized by the presence of the so-called “integument.” The early stages in the formation of the integument have not been observed, but from the available material certain facts with respect to its origin and development may be deduced. That it was not formed until the sporangium had attained, or almost attained, maximum size is evident from the dimensions of some of the young unintegumented specimens. That it had its origin in the lateral margins of the pedicel of the sporophyll is indicated by a comparison of the relative position of the tissues, vascular strand and parichnos, in the young and old specimens.

The integument is a multicellular layer thicker than the wall of the sporangium (figs. 34, 35). It is composed of some fifteen to eighteen rows of vertically elongated cells bounded by an epidermis of smaller and isodiametric cells. There is no trace of any vascular tissue, nor are there any sclerenchymatous cells. The integument, therefore, would not be a very rigid layer.

Figure 34 is a detail of a portion of the megaspore membrane, the wall of the sporangium, and the integument, showing their relation to one another. A comparison of the megaspore membrane and the sporangium wall of this integumented specimen with those of a young sporangium (12, fig. 9) reveals for the older sporangium a thinner megaspore membrane but a much thicker sporangial wall. These differences demonstrate a progressive evolution and elaboration of the tissues surrounding the megaspore that is not terminated with its maturation but persists with the development of the megagametophyte and the formation of the integument.

Among the many specimens of *Lepidocarpon* found in coal ball H 227 are two unusually well preserved integumented ones, drawn to the same scale in figures 31



FIGS. 33-35. *Lepidocarpon lomaxi*: Fig. 33 A, diagram of basal portion of tangential section of immature *Lepidocarpon* showing position of vascular strand. B, detail of vascular strand of A. Fig. 34, detail of portion of megaspore membrane, wall of sporangium, and integument from mature specimen. Fig. 35, median tangential section of the *Lepidocarpon* shown diagrammatically in fig. 32. Noé coll. H 227 B 12.

and 32. The dimensions of height and width of the two, respectively, are 9 by 11 mm. and 3.5 by 3 mm. Such a discrepancy in size of specimens of apparently the same age would make it difficult to reduce them to the same species, were it not for another feature that suggests an alternative explanation. The megaspore membrane of the larger is normal in position and thickness for the integumented stage in the ontogeny of *Lepidocarpon*; in the smaller there is a remnant of the membrane in the basal region of the sporangium (fig. 35mm), as if there had been an incipient development of the megaspore and consequent abortion of the megagametophyte. It is suggestive of an unpollinated ovule in a strobilus of *Zamia*; in such an ovule there is cessation of development, with final disintegration of the megagametophyte or endosperm, and at the same time continuous but less extensive development of the surrounding sporophyte tissues, nucellus, and integument.

### Summary

1. These studies were based on coal ball material collected by Dr. A. C. Noé of the University of Chicago. The coal balls were found near Harrisburg, Illinois, in coal no. 5, in the Alleghany group of the Upper Pennsylvanian.

2. The genera described are both vegetative and reproductive organs belonging in the *Lepidodendrales* order.

3. The genera possessing specific diagnostic features either are harmonized with established species or are described as new; those represented by a single specimen only, or those with insufficient preservation, and therefore inadequately known, are described and recorded under the generic name.

4. The genera and species described are: *Lepidophyllum aciculum* sp. nov.; *L. trichosulcata* sp. nov.; *L. alatum* sp. nov.; *L. latifolium* Graham; *L. thomasi* Graham; *Sigillariopsis cordata* sp. nov.; *Stigmara ficoides* Br., and stigmarian rootlets; *Lepidostrobus* Br. cone axis, cone scale, and microsporangium with tetrads of spores; *Triletes* Reinsch (megaspores of *Lepidodendron*), four types; *Lepidocarpon lomaxi* Scott.

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# DEVELOPMENT OF SEEDLINGS OF HETEROPOGON CONTORTUS AS RELATED TO SOIL MOISTURE AND COMPETITION

GEORGE E. GLENDENING

(WITH FOUR FIGURES)

## Introduction

On the grazing ranges of southwestern United States many important forage grasses reproduce from seed only during exceptionally favorable years, although there may continually be a supply of viable seed in the soil. Germination normally occurs during July or August, as a result of soil moistening by sporadic rains characteristic at that time. High temperatures and low relative humidity lead to rapid drying of surface soils; consequently conditions favorable to germination and seedling growth occur only during intermittent short periods.

This study of seedling development of a typical range grass, tanglehead (*Heteropogon contortus* (L.) Beauv.), was made under range conditions during the summer of 1938 and in a greenhouse during the winter of 1938 and spring of 1939. Tanglehead is a coarse, perennial bunchgrass of wide distribution (5). In the United States it is of local importance in the warmer and drier parts of Texas, New Mexico, and Arizona. The field study was designed to determine the rate and manner of shoot and root development under natural soil and climatic conditions, while the objectives of the greenhouse study were to determine, under partially controlled conditions, the effects of soil moisture and of competition upon the development of shoots and roots and survival or death of the seedlings during periods of drought.

Other studies (1, 7, 16) have shown that later reactions of grasses, including resistance to drought and winter injury, are closely associated with the amount of growth, particularly root development, made during seedling stages. Soil moisture has been shown (19, 20, 21) to affect top and root growth of grasses, with maximum root development occurring under moisture conditions below the optimum for top growth. It has also been shown (17, 20) that extent of root development in grasses varies with soil texture, greatest penetration and widest spread occurring in loose soils as compared with that in compact soils. Investigations (11, 13, 14, 15, 20) have indicated that the effects of competition among plants for various factors of the habitat, with respect to reduction of top and root growth, are consistent and subject to quantitative measurement.

## Methods of study

### IN THE FIELD

The field study plot was located on the Santa Rita Experimental Range, south of Tucson, Arizona, at an altitude of 3000 feet. The vegetation, representing a transition from desert plains grassland to southern desert shrub, is composed mainly of trees and shrubs, including *Prosopis velutina*, *Acacia greggii*, *Aplopappus fruticosus*, *Calliandra eriophylla*, *Krameria glandulosa*, and others. Numerous artificial revegetation tests have indicated that this area represents approximately the lower range of perennial grass stands. At present such grasses as *Bouteloua rothrockii*, *B. chondrosioides*, *B. filiformis*, *B. curtipendula*, *Aristida divaricata*, *A. glabrata*, *Hilaria belangeri*, *Trichachne californica*, *Triodia mutica*, *T. pulchella*, and others occur locally on the better sites.

The soil on this area has been classified as Continental gravelly loam (22). At the surface it is a sandy loam with a small amount of red clay. At approximately 12 inches the clay content increases, and a layer of loose small stones is encountered. Samples from the 6-inch level have a moisture equivalent of 6.587 per cent and a wilting coefficient, as determined indirectly (2), of 3.58 per cent.

High summer temperatures, low relative humidity, and distinctly seasonal rainfall are characteristic. Records taken at the area during the 5-year period preceding this study show that mean maximum air temperatures varied from 98° F. in July to 61° F. in January, while the mean minima for these two months were 72° and 34° F., respectively. Summer temperatures frequently exceed 100° F., while freezing temperatures occur only occasionally during the winter months. Monthly mean maximum soil temperatures (measured at the 6-inch level) varied from 102° F. in June to 49° F. in January, while the minima were 84° and 38° F., respectively, for the same months. Maximum surface soil temperatures of 150° F. have been recorded on this area. During the 5-year period, the average mean maximum wet-bulb depression for the months of June, July, August, and September was 30° F., and the total annual evaporation from a free-water surface (shallow tank) was in excess of 100 inches, or approximately eight times the total annual precipitation of 12.40 inches. There are two distinct rainfall seasons and two dry periods. Approximately 60 per cent of the total annual rainfall occurs during the months of July, August, and September, and about 25 per cent falls during December, January, and February. A spring dry period occurs characteristically during April, May, and June, and the month of October is usually dry. Climatic records taken during July, August, and September of 1938 indicate that conditions for plant growth were fairly typical during the course of the study, but the season was a little cooler and drier than the average for the preceding 5-year period.

On July 12, 1938, a plot 8×16 feet, within a cattle and rabbit enclosure, was



planted with tanglehead seed. As a result of two periods of favorable rainfall, crops of seedlings emerged on July 23 and August 6. At 2- to 4-day intervals, seedlings from the two crops were excavated, and the following observations and measurements taken: number of leaves and length of longest leaf; length of primary root and of its laterals; number and length of adventitious roots and length of lateral roots from them. Altogether, 101 seedlings were examined during a 49-day period. At each excavation, samples for soil-moisture determinations were taken at the surface-inch, 6-inch, and 12-inch level. Moisture determinations were made by standard desiccation methods.

#### IN THE GREENHOUSE

This phase, conducted at the University of Arizona, included the observation and measurement of tanglehead seedlings grown in prepared soil in pots under four varying amounts of moisture and three different intensities of competition.

On November 11, 1938, seed was planted in ninety-three no. 2 glazed pots in a homogeneous soil composed of two parts of clean river sand and one part of silt loam, the mixture having a moisture equivalent of 6.197 per cent. On November 21 the pots were divided into a W series and an S series for treatment under varying amounts of moisture and different intensities of competition, respectively. The W series was further divided into four groups, WA, WB, WC, and WD, of thirteen pots each, watered at intervals of 10, 4, and 2 days, and at irregular intervals, respectively. Each pot contained ten seedlings. The S series was divided into three groups, SA, SB, and SC, of thirteen pots each, with twenty, ten, and four seedlings per pot, respectively. These pots were watered at 4-day intervals. All pots received one-half pint of water at each irrigation.

The location of the pots on the greenhouse bench and the sequence of examination were randomized (18). The plants were grown for 6 weeks, during which the WA, WB, WC, and WD pots received the equivalent of 1.4, 2.9, 5.7, and 2.6 surface inches of water, respectively, while all pots under the competitive treatments (S series) received 2.9 inches.

Beginning November 27, 1938, and at twice-weekly intervals until January 2, 1939, the plants in one pot under each treatment were washed out for observation. Just prior to this removal of the plants, samples of soil for moisture determinations were obtained.

On January 2, irrigation of the remaining three pots under each treatment was discontinued. On February 14, the plants in one pot from each treatment were washed out to determine the growth made before drought dormancy set in, and on the same day irrigation of a second pot from each treatment was begun to determine whether or not the plants would resume growth. The third set of plants,

one pot from each treatment, was allowed further to dry out until March 17, when water was applied.

In addition to observations made in the field study, dry weights of shoots and roots were determined, and photographs of representative plants were obtained at critical periods.

Air temperature in the greenhouse was regulated thermostatically and was further controlled by manipulation of ventilators. Usually temperatures were lower and relative humidity greater than on the field plot, while sunlight intensity and length of day were less. On the whole the character of growth made by the seedlings was similar to that on the field plot.

### Results

#### IN THE FIELD

Growth data for the two crops of seedlings are summarized in table 1, and drawings of representative plants are shown in figures 1 and 2. Growth was slow at first and mainly confined to elongation of the primary root. When the first leaves emerged, the primary root was slightly over 1 inch in length and directed vertically downward. It continued to elongate vertically for approximately 30 days. The primary root system consists of only the primary root with its laterals; no other seminal roots are developed. The maximum length measured was 10.4 inches, but the greatest average length of the primary roots of any group of seedlings was 6.4 inches. Lateral roots appeared after the third day and reached an average length of about 1 inch at 18–20 days. Most of these were formed in a zone 2–3 inches below the soil surface. After 20–30 days the cortex was invariably sloughing on the primary roots, the laterals were dry and fragile, and the growing points had apparently ceased to elongate.

Adventitious or nodal roots appeared 15 and 23 days after emergence on the July 23 and August 6 plants, respectively, but were organized somewhat earlier since plants left in water over night on the twelfth day developed short adventitious roots. A comparison of the data in tables 1 and 2 shows that in each crop of seedlings adventitious roots were first recorded a few days after rainfall had resulted in available moisture in the surface soil.

Tillering seemed definitely associated with development of adventitious roots. This agrees with the work of others (6, 19). Leaf number increased slowly at first and then rather rapidly following development of nodal roots. At the close of the study most plants had two or three tillers and a few of the largest had four.

#### IN THE GREENHOUSE

VARIATION IN MOISTURE.—Table 3 shows that frequency of watering was reflected directly in moisture content and relative wetness (3) of the soil in the pots.

Average relative wetness of the soil was 84, 111, 168, and 111 in the WA, WB, WC, and WD pots from November 27 to January 2. An obvious difference resulting from this variation in moisture supply was that of number and length of leaves.

TABLE 1  
GROWTH MEASUREMENTS (IN INCHES) ON FIELD-GROWN SEEDLINGS

DATE		NO. OF DAYS AFTER EMER- GENCE	LEAVES		PRIMARY ROOT SYSTEM		ADVENTITIOUS ROOT SYSTEM		
			NUMBER	LENGTH OF LONGEST	LENGTH OF PRI- MARY ROOT	LENGTH OF LATERALS	NO. OF ROOTS	LENGTH OF ROOTS	LENGTH OF LATERALS
CROP OF JULY 23, 1938									
July	24.....	I	I	0.3	I.2	Trace			
	26.....	3	2	0.3	1.6				
	28.....	5	2	0.3	2.7				0.1
August	I.....	9	3	0.4	3.2	0.5	I	Trace	
	4.....	12	3	0.5	3.2	0.5			
	7.....	15	4	0.7	3.5	0.7			
	11.....	19	6	0.9	4.3	0.8			
	16.....	24	6	I.5		2			0.1
	23.....	31	II	2.1		2			0.5
	26.....	34	14	2.5		3			2.2
September	7.....	46	24	3.7			4	6.4	I.0
							6	4.1	3.0
CROP OF AUGUST 6, 1938									
August	7.....	I	I	0.3	I.2				
	11.....	5	2	0.4	1.9				0.2
	14.....	8	3	0.5	2.5				0.4
	16.....	10	4	0.4	2.7				0.5
	19.....	13	5	0.6	3.4				0.6
	24.....	18	7	1.3	5.3				1.0
	29.....	23	14	1.3	6.4				
September	I.....	26	17	1.7		Trace	Trace	0.5	
	4.....	29	17	1.9		4	1.8		
	7.....	32	19	3.2		4	3.7		I.0
	11.....	36	22	2.8		4	4.0		I.0
						5	4.8		3.0

This difference was small at first (table 4) but increased with age. On January 2, 44 days after emergence, both length and number of leaves were directly proportional to frequency of irrigation.

The rate of elongation of the primary roots was about equal under all treatments for approximately 30 days. The primary roots in the WC and WD plants elongated very little after December 20, however, while those of the WA and WB

plants were still growing on January 2. Maximum lengths attained were 15.11, 15.47, 12.31, and 13.40 inches in the order WA to WD. Lateral roots were similar in length in the WA, WB, and WD plants but were more numerous on the latter two than on the least watered (WA) plants. They were most numerous and of greatest length on the most watered (WC) plants.

Adventitious roots appeared on the WC plants on December 4, 15 days after emergence, and on the WB and WD plants 19-23 days after emergence (table 5). Development of adventitious roots in the WA plants was meager. Adventitious

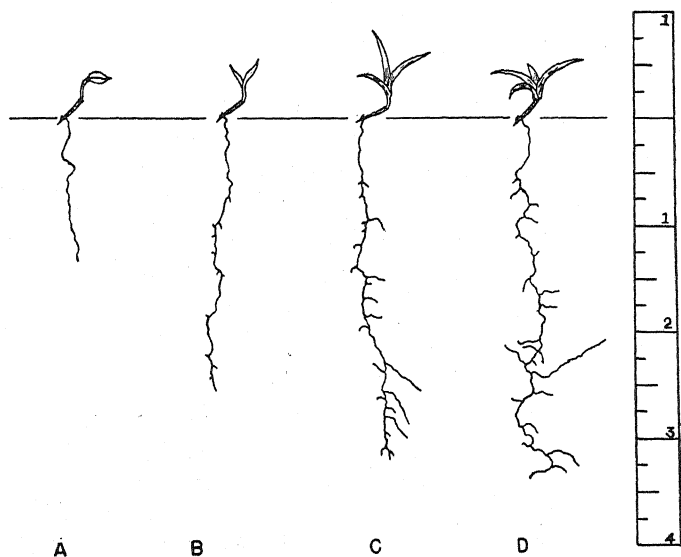


FIG. 1.—Early stages in development of tanglehead seedlings on field plot. Plants A to D excavated 1, 5, 9, and 12 days, respectively, after emergence of shoots.

root laterals were few to entirely absent on the WA and most numerous on the WC plants. Sequence and extent of tillering varied directly with frequency of watering. Representative plants from each of the treatments are shown in figure 3.

The dry weights of shoots on January 2 were 0.034, 0.056, 0.090, and 0.074 gm. per plant, and those of roots (total) 0.02, 0.038, 0.036, and 0.041 gm. under the WA, WB, WC, and WD treatments, respectively. These show that weight of shoots was directly proportional to irrigation frequency, and that root weights followed a similar but less definite trend. Shoot/root ratios on January 2 were 1.70:1, 1.47:1, 2.50:1, and 1.80:1 in the WA, WB, WC, and WD plants.

**COMPETITION.**—The effect of variation in number of plants per pot upon seedling development was less pronounced than that of variation in frequency of watering (table 6). For approximately 30 days leaf growth varied directly with

denseness of planting. By January 2, however, this condition was reversed and the lengths were 5, 5.30, and 5.86 inches in seedlings grown at the rate of twenty, ten,

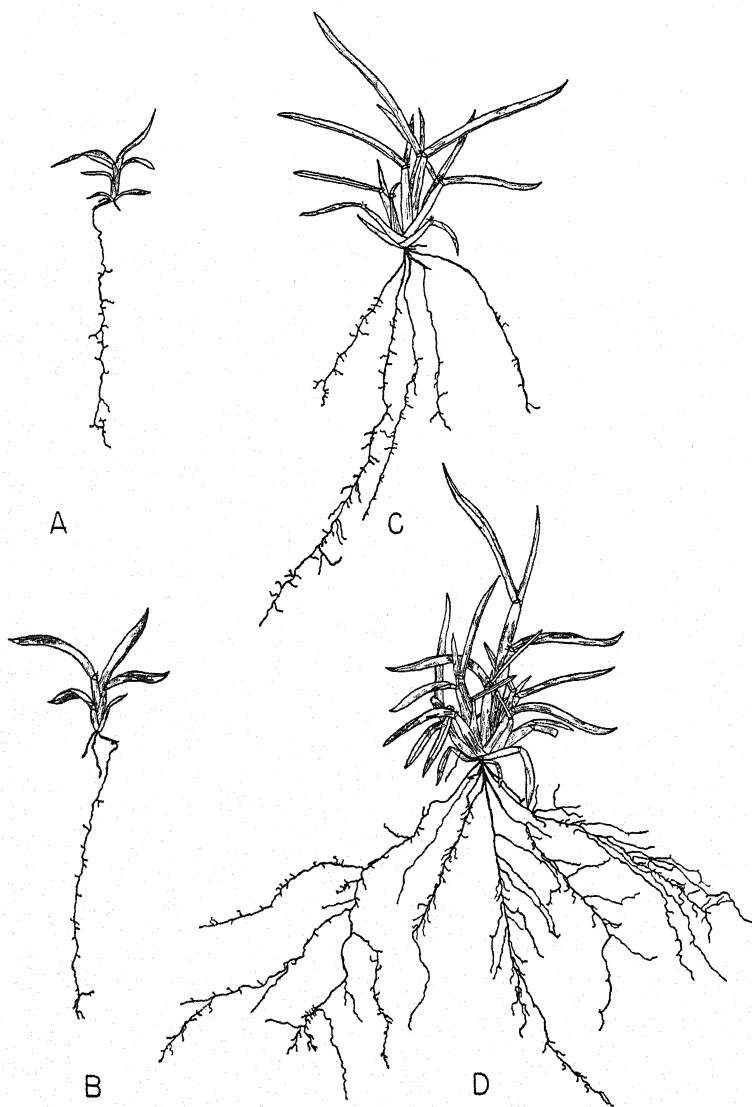


FIG. 2.—Later stages in growth of seedlings on field plot. Plants *A* to *D* excavated 19, 24, 34, to 45 days after emergence. Plant *D* is slightly above average in size.

and four plants per pot. Density of planting had no significant effect on the number of leaves and amount of tillering.

On January 2, lengths of the primary roots were 12.27, 15.47, and 14.13 inches

in the SA, SB, and SC pots. Primary root laterals were fewest in the SA plants and mainly confined to the lower portions of the root. They were more numerous and more generally distributed in the SB and SC plants. Adventitious roots appeared

TABLE 2  
RAINFALL, PERCENTAGE SOIL MOISTURE, AND RELATIVE WETNESS\* ON FIELD PLOT

DATE	RAINFALL (INCHES)	SURFACE INCH		6-INCH LEVEL		12-INCH LEVEL	
		SOIL M. (%)	REL. WET.	SOIL M. (%)	REL. WET.	SOIL M. (%)	REL. WET.
July	11.....	0.01	.....	.....	.....	.....	.....
	17.....	0.15	.....	.....	.....	.....	.....
	20.....	1.03	.....	.....	.....	.....	.....
	21.....	.....	6.8	103	7.1	108	6.1
	22.....	0.02	.....	.....	.....	.....	.....
	23.....	0.04	.....	.....	.....	.....	.....
	24.....	.....	3.3	50	6.0	91	6.2
	26.....	.....	0.7	11	5.4	82	5.5
	28.....	.....	0.4	6	4.8	73	.....
August	1.....	.....	0.2	3	3.7	56	4.9
	3.....	0.17	.....	.....	.....	.....	.....
	4.....	0.51	0.8	12	3.1	47	4.6
	6.....	2.02	.....	.....	.....	.....	.....
	7.....	.....	8.0	121	10.0	152	10.1
	9.....	.....	4.1	62	6.8	103	8.3
	11.....	.....	0.7	11	5.7	86	.....
	12.....	0.98	.....	.....	.....	.....	.....
	14.....	.....	6.3	95	8.2	124	8.6
	16.....	.....	1.4	21	6.9	104	7.1
	19.....	.....	0.02	0.3	5.3	80	4.4
	24.....	0.14	5.0	76	3.8	58	4.7
	25.....	0.14	.....	.....	.....	.....	.....
	26.....	.....	1.0	15	4.5	68	5.1
	28.....	0.04	.....	.....	.....	.....	.....
	29.....	.....	0.4	6	4.9	74	5.2
	30.....	0.30	.....	.....	.....	.....	.....
September	1.....	.....	0.8	12	4.9	74	4.8
	2.....	0.20	.....	.....	.....	.....	.....
	4.....	.....	2.3	35	4.9	74	4.9
	5.....	0.37	.....	.....	.....	.....	.....
	7.....	.....	1.6	24	6.5	98	6.3
	11.....	.....	0.3	5	5.1	77	5.2

\*Percentage soil moisture divided by moisture equivalent, the quotient  $\times 100$ , as suggested by CONRAD and VEIHMAYER (3).

after about 3 weeks, and in all cases rapid elongation of the primary root system appeared to cease.

Adventitious roots were most numerous under the medium dense spacing (SB) and fewest on the plants (SA) grown under the greatest competition (table 7). The rate of elongation was similar under all degrees of competition. On January 2, lengths of adventitious roots (8.66, 8.33, and 8.15 inches) varied directly with denseness of planting, while lengths of adventitious laterals (1.11, 1.75, and 1.92

inches) were inversely related to degree of competition. Representative plants photographed on January 2 are shown in figure 4.

TABLE 3

PERCENTAGE SOIL MOISTURE AND RELATIVE WETNESS IN POTS UNDER VARIOUS TREATMENTS DURING PERIOD OF IRRIGATION

DATE	WA		SB-WB*		WC		WD		SA		SC	
	SOIL M. (%)	REL. WET.	SOIL M. (%)	REL. WET.	SOIL M. (%)	REL. WET.	SOIL M. (%)	REL. WET.	SOIL M. (%)	REL. WET.	SOIL M. (%)	REL. WET.
Nov. 27.....	6.3	102	5.7	92	9.0	145	6.9	111	5.2	84	4.6	74
30.....	4.6	74	6.6	106	6.0	97	7.2	116	7.8	126	6.9	111
Dec. 1.....	5.7	92	7.2	116	9.1	147	7.9	127	6.6	106	4.9	79
4.....	4.7	76	7.8	126	10.2	165	7.9	127	6.1	98	6.9	111
8.....	6.8	110	6.9	111	11.8	190	5.5	89	4.5	73	4.9	79
10.....	4.8	77	7.8	126	8.0	129	6.3	102	4.3	69	6.7	108
12.....	5.5	89	5.6	90	7.7	124	7.3	118	6.5	105	7.7	124
15.....	7.3†	118	6.1	98	8.5	137	7.8	126	6.4	103	8.4	135
20.....	5.5	89	8.0	129	11.4	184	8.6	139	7.0	113	6.9	111
23.....	5.3	85	7.2	116	14.7	237	6.3	102	7.2	116	6.8	110
27.....	4.7	76	7.2	116	14.4	232	6.0	97	7.6	123	7.9	127
30.....	3.9	63	7.6	123	10.2	165	5.6	90	6.0	97	5.4	87
Jan. 2.....	4.4	71	5.7	92	14.4	232	5.9	95	5.4	87	5.8	94
Mean....	5.2	84	6.9	111	10.4	168	6.9	111	6.2	100	6.5	105
High....	6.8	110	8.0	129	14.7	237	8.6	139	7.8	126	8.4	135
Low.....	3.9	63	5.6	90	6.0	97	5.5	89	4.3	69	4.6	74

\* Average of two pots.

† Watered by mistake and not included in average.

TABLE 4

GROWTH OF LEAVES AND PRIMARY ROOT SYSTEM AS AFFECTED BY FREQUENCY OF WATERING

DATE*	AVERAGE LENGTH (INCHES)											
	WA			WB			WC			WD		
	LEAVES	PRIM- ARY ROOT	LAT- ERALS	LEAVES	PRIM- ARY ROOT	LAT- ERALS	LEAVES	PRIM- ARY ROOT	LAT- ERALS	LEAVES	PRIM- ARY ROOT	LAT- ERALS
November 30-December 4.....	1.00	5.70	1.75	2.38	4.79	1.62	2.18	5.25	1.50	2.74	7.93	2.00
December 8-12†.....	3.01	9.24	2.84	3.45	10.72	2.45	3.11	9.62	3.29	2.61	9.61	2.72
December 15-20.....	3.97	11.31	.....	4.26	11.67	.....	5.97	12.07	.....	5.14	12.66	.....
December 23-27.....	4.00	14.64	.....	4.45	15.01	.....	8.03	12.31	.....	6.15	13.20	.....
December 30-January 2.....	3.90	15.11	.....	5.30	15.47	.....	7.07	11.55	.....	6.06	13.40	.....
No. of leaves January 2 .....	(4.4)			(6.6)			(8.0)			(6.8)		

\* Figures represent average of measurements on the two dates shown.

† No measurements of lateral roots made after this date.

On January 2 the average dry weight of roots per plant was 0.027, 0.038, and 0.041 gm. under the SA, SB, and SC treatments, while shoot weights were 0.051, 0.056, and 0.057 gm., showing that the weight of shoots and roots was inversely

related to the degree of competition. Shoot/root ratios in the same order were 1.88:1, 1.47:1, and 1.39:1, indicating greatest relative shoot production under the most intense competition.

FINAL GROWTH AFTER DISCONTINUED WATERING.—The moisture in all the pots soon after watering ceased was below the wilting coefficient (3.37 per cent) in all

TABLE 5  
DEVELOPMENT OF ADVENTITIOUS ROOT SYSTEM AS AFFECTED BY FREQUENCY OF WATERING

DATE*	No. OF ROOTS				LENGTH OF ROOTS (INCHES)				LENGTH OF LATERAL ROOTS (INCHES)			
	WA	WB	WC	WD	WA	WB	WC	WD	WA	WB	WC	WD
November 30–December 4.....	.....	.....	0.80	.....	.....	.....	1.32	.....	.....	.....	.....	.....
December 8–12.....	.....	0.30	1.00	0.30	.....	1.69	1.98	0.25	.....	.....	.....	.....
December 15–20.....	0.55	1.65	1.40	1.60	1.05	1.74	6.57	4.70	.....	0.35	0.98	0.44
December 23–27.....	0.40	1.70	2.65	1.40	0.23	6.21	6.12	8.01	.....	0.76	2.87	1.61
December 30–January 2....	0.40	2.05	2.60	1.80	2.85	8.33	8.34	10.48	0.15	1.75	4.63	1.26

\* Figures represent average of measurements on the two dates shown.

TABLE 6  
GROWTH OF LEAVES AND PRIMARY ROOT SYSTEM AS AFFECTED BY COMPETITION

DATE*	AVERAGE LENGTH (INCHES)								
	SA			SB			SC		
	LEAVES	PRIMARY ROOT	LAT- ERALS	LEAVES	PRIMARY ROOT	LAT- ERALS	LEAVES	PRIMARY ROOT	LAT- ERALS
November 30–December 4.....	2.52	5.66	1.87	2.38	4.79	1.48	2.16	7.13	0.81
December 8–12†.....	3.06	11.85	2.55	3.45	10.72	2.45	2.96	10.68	3.62
December 15–20.....	4.77	10.65	.....	4.26	11.67	.....	3.98	11.14	.....
December 23–27.....	4.90	12.21	.....	4.45	15.01	.....	4.46	13.26	.....
December 30–January 2.....	5.00	12.27	.....	5.30	15.47	.....	5.86	14.13	.....
No. of leaves January 2.....	(5.3)			(6.6)			(5.7)		

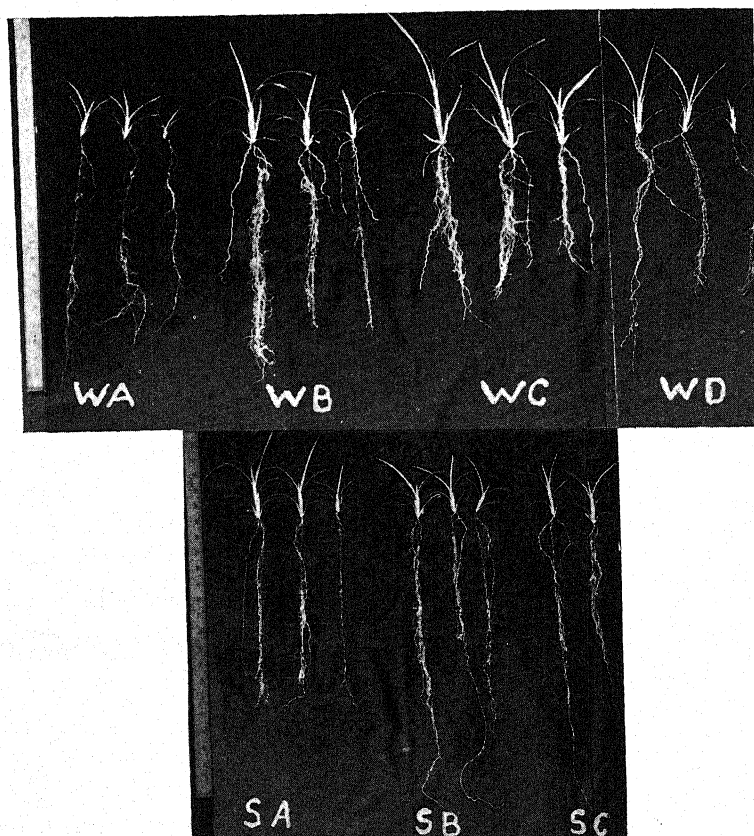
\* Figures represent average of measurements on the two dates shown.

† No measurements of laterals made after this date.

samples taken after January 25, indicating that a serious moisture shortage existed from January 25 to February 14. At the end of the 6-week dry period all the plants were completely dormant, with those in the most frequently watered (WC) and least densely planted (SC) pots appearing to be drier than those grown with less moisture and under more intense competition. One set of plants on February 14 showed that leaf growth made before dormancy set in was slight in the WA and



WD, somewhat greater in the WB, and much greater in the WC plants. Under all treatments, with the exception of the SC plants, where the number of leaves tripled, the number of leaves on February 14 was approximately double that on January 2.



FIGS. 3, 4.—Fig. 3 (above), largest-, smallest-, and average-sized plants from pots watered at 10-, 4-, 2-day, and irregular intervals, in order from left to right. Fig. 4 (below), representative seedlings from pots receiving equal amounts of water but containing 20, 10, and 4 plants, respectively, from left to right. Top growth of SA plants is above average in size, but root growth is typical.

Primary root systems, with the exception of those of the WA plants which had elongated to 16.75 inches, were badly disintegrated, and no measurements other than weight were made. With few exceptions the number, length, and length of laterals of the adventitious roots on the plants examined 6 weeks after irrigation ceased were directly proportional to the frequency at which the plants had previously been watered and inversely proportional to the number per pot. The final dry weights of shoots and roots showed the same relationship. Shoot/root ratios

were not consistent as affected by moisture but tended to increase with the amount of water applied, while with respect to competition the shoot/root ratios were inversely proportional to the number of plants per pot.

Resumption of growth by the plants when watered after the dry-up period of 6 weeks was rapid. Most plants showed some greening of leaves within 24 hours and on the second day were again growing rapidly. New growth was initiated from the axial buds of the basal leaves, and in most cases the old leaves became active. Under all treatments at least some of the old leaves regained their turgor and became green. Flowering occurred in the WD plants only 7 days after watering was resumed. The remaining plants then flowered in the order SB, WB, SC, WC, SA,

TABLE 7

DEVELOPMENT OF ADVENTITIOUS ROOT SYSTEM AS AFFECTED BY COMPETITION

DATE*	NO. OF ROOTS			LENGTH OF ROOTS (INCHES)			LENGTH OF LATERAL ROOTS (INCHES)		
	SA	SB	SC	SA	SB	SC	SA	SB	SC
November 30-December 4.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
December 8-12.....	0.10	0.30	0.25	1.52	1.69	3.05	.....	.....	.....
December 15-20.....	1.30	1.65	1.40	3.48	1.74	1.90	0.43	0.40	1.20
December 23-27.....	1.55	1.70	1.50	7.09	6.21	7.62	0.84	1.16	0.90
December 30-January 2.....	1.35	2.05	2.00	8.66	8.33	8.15	1.11	1.75	1.92

\* Figures represent average of measurements on the two dates shown.

and WA, with the last plants in the anthesis stage on March 22, 36 days after watering was resumed.

As compared with the recovery at the close of the 6-week dry period, resumption of growth after 11 weeks of drought was slow, although there was noticeable greening of old leaves in the SC plants within 24 hours after being watered, and by the fourth day there were new leaves from basal buds on the WA, WD, SB, and WB plants. There was no sign of renewed growth in the SA and WC plants, and examination showed them to be dead. In the plants which had been grown under the least water (WA and WD), many of the old leaves became green.

### Discussion

The rate of growth of the field-grown seedlings during the first 40 days after emergence was relatively slow, although soil moisture at the 6-inch level was above the wilting coefficient and therefore, according to CONRAD and VEIHMEYER (3), sufficient to produce normal growth at all times. The relatively slow growth rate of perennial grasses has been pointed out by PARKER and SAMPSON (12). Despite

their small size at the end of the experiments, the seedlings remained alive during the fall dry period which followed.

Data from both the range and the greenhouse indicate that moisture content of the soil is probably the major controlling factor in the development of these plants under range conditions. Depth of penetration of primary roots and the zone of maximum lateral root development correspond closely with the depth to which water from the periodic showers penetrates.

Under field conditions the initiation of adventitious or permanent roots is directly controlled by surface soil moisture. This was also the case in the greenhouse, where plants grown in pots in which the surface soil was usually dry developed an average of less than one adventitious root per plant during 6 weeks of growth. LOCKE and CLARK (10) noted a similar condition, wherein wheat grown in Oklahoma under severe drought conditions did not develop adventitious roots.

In all cases where the tanglehead seedlings developed adventitious roots, the primary roots soon ceased to elongate and became very brittle. There is some disagreement in the literature as to the function of primary roots in grasses after the adventitious root system is established. HAYWARD (4) suggests that the primary roots of corn are of little importance after the adventitious root system is established, and LA RUE (9) indicates the same with regard to wheat, barley, and other grasses. KRASSOVSKY (8), however, states that the seminal roots of summer wheat, barley, and rye remain functional up to the time of harvest. It was impossible to determine definitely whether or not the primary roots of the tanglehead seedlings ceased to function after the adventitious roots were established, but it is significant that in plants where few or no adventitious roots developed, the primary roots remained intact and definitely elongated up to the final set of observations.

The relatively meager development of shoots as compared with roots in tanglehead seedlings is probably significant. In general the ratio of shoots to roots increased in direct proportion to the amount of water applied, which is in agreement with the results of other studies (15, 19). These ratios, based on air-dry weight, varied from 1.7:1 to 2.5:1 and are very low compared with those reported for Sudan grass (11), which is considered a relatively drought-resistant species. The value of a large absorbing system coupled with a small transpiring surface in plants, which under natural conditions are frequently subjected to adverse moisture conditions, is hardly to be questioned.

Denseness of planting appears to be of minor importance during the early stages of seedling development and while soil moisture is available. When moisture becomes limiting, thickly planted seedlings are at a definite disadvantage as compared with those growing under less crowded conditions and having a lower total moisture requirement per unit of soil. Seedlings planted at the rate of

twenty per pot failed to resume growth after a drought period of 11 weeks, whereas those grown at the rate of four per pot became green within 24 hours after being watered, and those planted at the rate of ten per pot became green after 48 hours. Similar responses have been noted on artificially seeded field plots, where densely planted seedlings appeared to make growth comparable with that of those planted less densely during the first growing season while moisture was available but suffered much greater drought losses during subsequent dry periods.

The fact that the old leaves of some plants regained their turgor and became active within 24 hours after water was applied following drought periods of 3 and 8 weeks, during which soil moisture was below the wilting coefficient, shows that the protoplasm of these leaves had not been permanently injured by the extremely unfavorable moisture conditions and is indicative of the inherent drought resistance of tanglehead seedlings.

### Summary

1. Based on dry weight, maximum development of shoots and roots of tanglehead grass seedlings occurred under most frequent irrigation and least intense competition. Shoot/root ratios were greatest under most frequent watering and most intense competition.
2. Lack of moisture at the soil surface retarded the initiation of adventitious roots under field and greenhouse conditions.
3. Tillering was closely associated with the establishment of the adventitious root system.
4. Soil moisture had a greater effect upon the plants than competition and appears to be the major controlling factor under range conditions.
5. Rapid resumption of growth after drought periods of 3 and 8 weeks, during which soil moisture was below the wilting coefficient, indicates the inherent drought resistance of these seedlings.

The writer is indebted to the members of the staff of the botany department of the University of Arizona for valuable suggestions and criticism. Especial mention is due Drs. D. M. CROOKS and R. A. DARROW, who helped plan the study, and JOSEPH F. ARNOLD for aid with the pictures.

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# MEGAGAMETOPHYTE DEVELOPMENT IN A TRIPLOID TULIP<sup>1</sup>

JOHN M. BELLOWS, JR., AND RONALD BAMFORD

(WITH FORTY-THREE FIGURES)

## Introduction

An earlier study (6) of the progeny resulting from reciprocal crosses of triploid ( $2n=36$ ) and diploid ( $2n=24$ ) tulips showed that the chromosome number of the functional gametes of the triploid is less than the expected number, if normal meiotic divisions, like those of the diploids, had taken place. The formation of the male gametes in triploids has been dealt with extensively (7, 8, 9, 13, 17, 25, 34, 38, 42, 48). The general conclusion seems to be that the functional gametes fail to receive the expected number of chromosomes, especially in the case of autotriploids, because of the chromosome association in the meiotic prophase. Separation of the chromosomes results in an unequal distribution, bridging and eventual fragmentation, lagging, and finally the loss of chromosomes during the meiotic or subsequent divisions. In addition to this there may also be a selection due to differential pollen-tube development and later to certain types of incompatibility. All these types of behavior explain the selection in favor of the diploid gametes, and in certain cases the tetraploid gametes (6, 26, 27, 29, 40, 46), which must function when the triploid is used as the pollen parent.

In contrast to the volume of publications concerning pollen formation in the triploid, there has been much less work on the meiotic and subsequent divisions during the formation of the megagametophyte in triploids. The most complete accounts of this development have been those of MORINAGA and FUKUSHIMA (30) on *Oryza*, SATINA and BLAKESLEE (39) on *Datura*, and WESTFALL (47) on *Lilium*. These all report the unequal and irregular distribution of chromosomes so frequently reported for meiosis in pollen formation. However, when the progeny of crosses involving the triploid as a seed parent are examined (6, 9, 17, 20, 23, 26, 27, 29, 32, 39, 46), they clearly indicate that a somewhat different type of behavior must occur during megagametophyte formation, because the female gametes usually have a higher chromosome content. DARLINGTON (16) has previously pointed this out. Our earlier studies (6) of  $3n \times 2n$  crosses, as compared with  $2n \times 3n$ , have shown that Inglescombe Yellow followed the behavior mentioned, and it is the purpose of this paper to discuss the relation the megagametophyte divisions have to this difference between the triploid as a pollen and as a seed parent.

<sup>1</sup> Contribution no. 713, Maryland Agricultural Experiment Station.

The type of megagametophyte division in diploid tulips has been described by BAMBACIONI and others (3, 4, 12, 18, 19), and there is little doubt that it falls in the category more recently described by COOPER and others (14, 15, 37, 47) for several species of *Lilium*. Extensive reviews concerning this type have been made by JOSHI (22) and SCHNARF (41). The latter has applied the term *Fritillaria*-type to such megagametophytes.

### Material and methods

Inglescombe Yellow, the triploid tulip selected for this study, is one of the *Leiostemones* group. UPCOTT (44) considers it a variety of *Tulipa gesneriana*, and—although it has proved to be self-sterile—it has been reciprocally crossed with certain diploids (6, 44, 49).

Collections of unopened buds from Maryland-grown bulbs were made in the field in the spring of 1938 and 1939. Additional collections of opened buds were made in the spring of 1940 from plants grown in the greenhouse. It was found that the final stages in the development of the megagametophyte do not take place until the flower has opened and the anthers are dehiscing. This study bears out the report of UPCOTT and LA COUR (45), however, that meiosis in the female gamete takes place just before the flower bud opens.

Fixations were made in Navashin's fluid. The material was treated with the butyl-alcohol technique and imbedded in Tissuemat. Sections were cut at 18–20  $\mu$  and stained with Heidenhain's iron-alum haematoxylin. Examination of the section was made with 16, 4, and 1.2-mm. apochromatic objectives and 15 $\times$  compensating oculars.

### Observations

The basic chromosome number in *Tulipa* is 12. Inglescombe Yellow, an autotriploid horticultural variety, was found to contain 36 chromosomes in the somatic cells of the early nucellar layer and in the surrounding cells of the ovule and ovary.

The megagametophyte arises by differentiation from a subepidermal cell which develops directly into the megaspore mother cell without undergoing further division. The megaspore mother cell increases in size until it is two to three times as long as wide. The nucleus increases in size to some extent with the cell. When both have attained maximum size, prior to the prophase stage of the first division, the cytoplasm of the cell is finely granular, very dense in appearance, and no large vacuoles are present (figs. 1, 19). One or more large nucleoli are usually found, in addition to many smaller nucleoli, in each nucleus. Immediately preceding the prophase stage the large nucleus of the megaspore mother cell will occupy a position in the center of the cell or slightly toward the micropylar end.

The first stage at which it was possible to determine the number and type of association among the chromosomes was at the early diakinesis of meiosis I (fig.

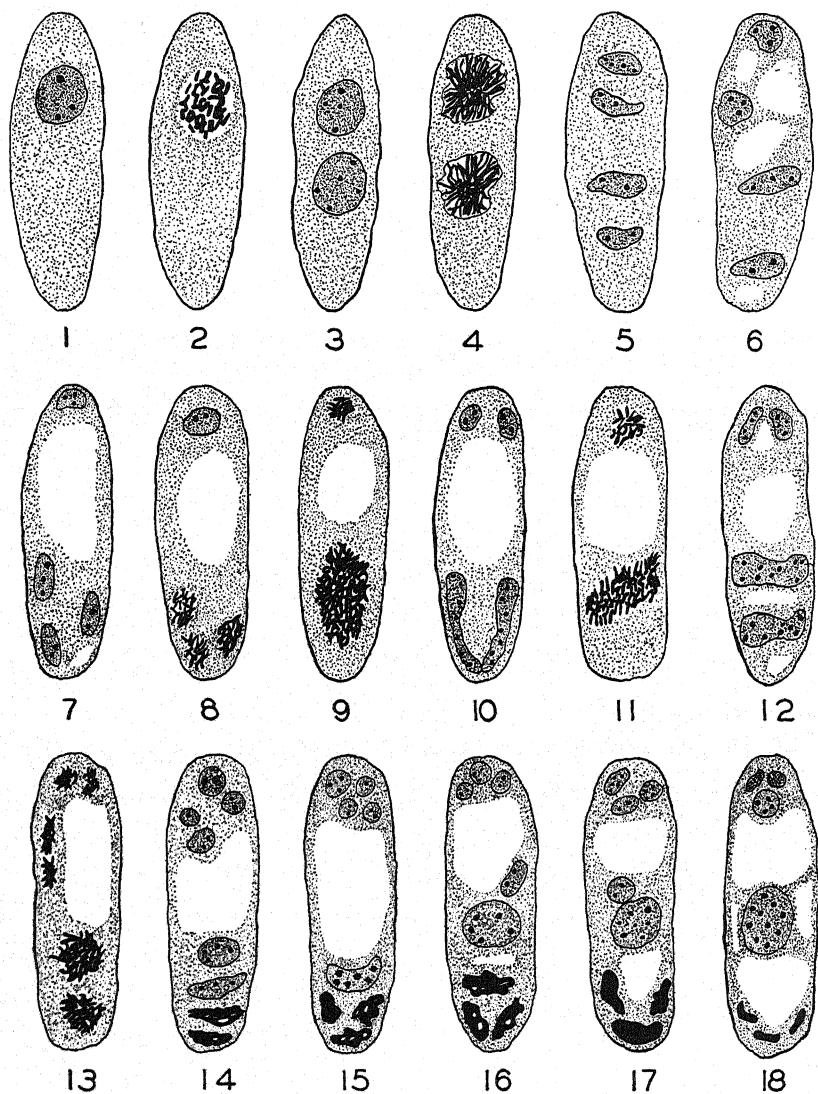
20). In one case a total of 6 trivalent and 9 bivalent chromosomes could be identified, but it was very difficult to obtain the total for all the cells observed. Later, during the formation of the metaphase plates for the first division, it was easier to determine the exact type of association. Again trivalents, bivalents, and univalents could always be identified (fig. 2). One metaphase plate contained 5 trivalents, 8 bivalents, and 5 univalents; another contained 6 trivalents, 6 bivalents, and 6 univalents; a third contained 6 trivalents, 5 bivalents, and 8 univalents.

Separation of the chromosomes in the anaphase stage seems to indicate that an 18-18 distribution is the most common (figs. 21, 22). Counts were made of one or both plates of this stage when possible. In one megasporocyte division a 19-17 distribution, and in another a 19-16 distribution, was observed. Only one division showed evidence of lagging chromosome material, and this was in the form of a chromosome bridge (fig. 22). As a result of this division, two nuclei with approximately the  $3n/2$  number of chromosomes are formed (figs. 3, 24), and micronuclei (fig. 23) are rare.

In the second meiotic division, an equational one, the spindles usually form in the same plane as that of the preceding, but occasionally they may form across or diagonally through the developing megagametophyte (figs. 25, 26). In one case both nuclei were in the metaphase stage and each contained 18 chromosomes. In two more both nuclei were in the anaphase stage. At the micropylar end of the first there was a 20-16 distribution, and at the chalazal end there was a 20-14 distribution. The other had a 20-15 distribution at the micropylar end and a 19-17 distribution at the chalazal end. It was difficult to make exact counts, but the distribution between the nuclei in many cases was approximately equal. Infrequently micronuclei appear in the cytoplasm after this division, indicating that some chromatin material has failed to be included in the four nuclei.

This division results in the formation of four megaspore nuclei, each with approximately 18 chromosomes, which align themselves throughout the length of the megagametophyte (fig. 5). After this division one of the nuclei at the micropylar end of the sac begins to migrate toward the two nuclei at the chalazal end (figs. 6, 27, 28), and as it approaches them the three move into a triangular position. A large vacuole partially separates the three at the chalazal end from the single one at the micropylar end (figs. 7, 29). Soon each of the three nuclei passes through the prophase stage, and three metaphase plates of chromosomes can be recognized (figs. 8, 30). The chromosomes from each of these three nuclei are then grouped in one large mass resembling a single metaphase plate, much larger than any one of the previous plates (figs. 9, 11, 31). A typical equational division follows in which each chromosome group takes part so that two equally large nuclei result (figs. 12, 34). The direction of the spindle alignment of this division at the chalazal end may be through the long axis of the megagametophyte or across it (figs. 31, 33).





FIGS. 1-18.\*—Fig. 1, megaspore mother nucleus, resting stage. Fig. 2, metaphase I. Fig. 3, two megaspore nuclei after meiosis I. Fig. 4, polar view of two dividing megaspore nuclei. Fig. 5, four megaspore nuclei after meiosis II. Figs. 6, 7, polarization of megagametophyte. Fig. 8, late prophase of three megaspore nuclei at chalazal end. Figs. 9, 11, third nuclear division. Figs. 10, 12, two triploid nuclei, chalazal end; two haploid nuclei, micropylar end. Fig. 13, fourth nuclear division. Figs. 14, 15, degeneration of antipodals. Fig. 16, migration of haploid nucleus. Fig. 17, formation of polar apparatus. Fig. 18, mature megagametophyte preceding fertilization.  $\times 450$ .

\* Diagrammatic drawings made either from photomicrographs or directly from microscope. Micropylar end of megagametophyte is pointed toward top of page in each case. "Figure incomplete" indicates that both ends of embryo sac were not included in one section.

The former arrangement appears more common. At the same time the nucleus remaining at the micropylar end also undergoes division. The spindle is generally aligned across the long axis of the megagametophyte (fig. 33) but may occasionally be formed at right angles to this plane.

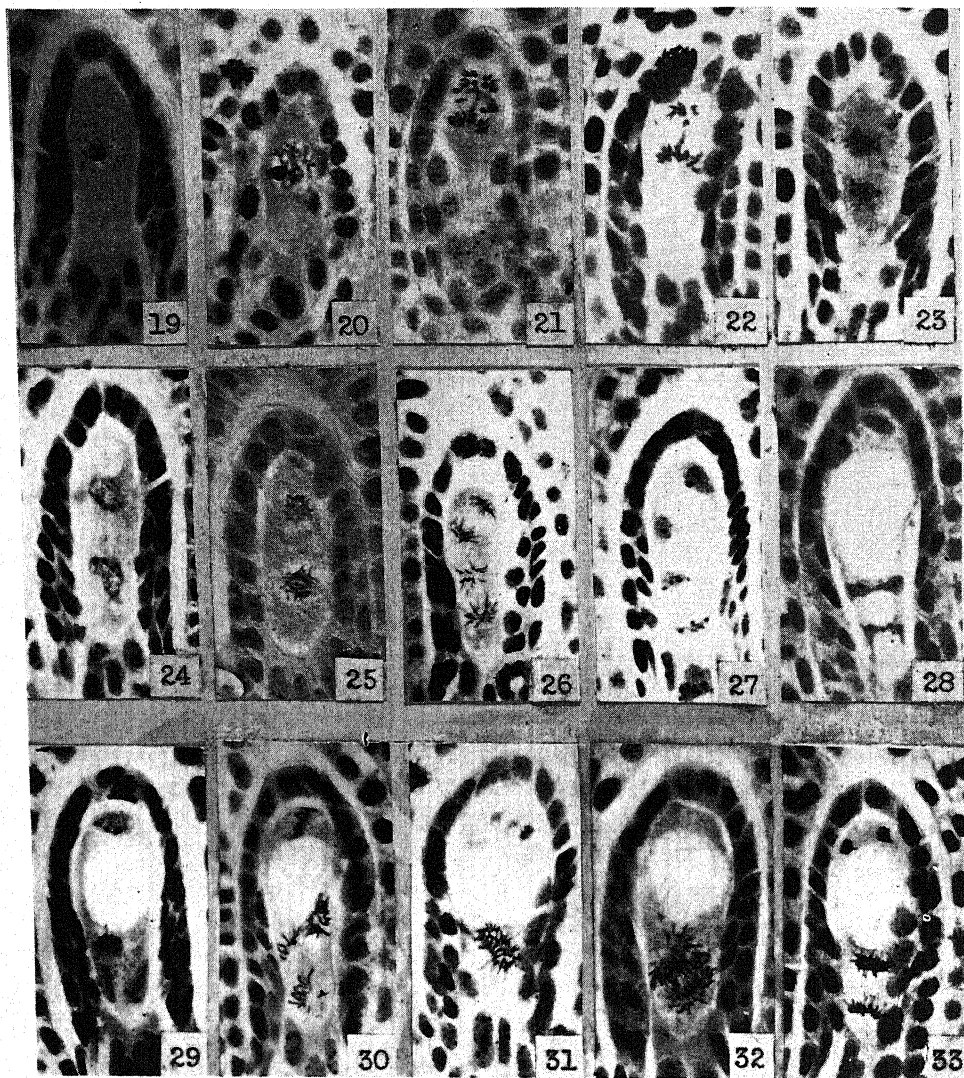
This third nuclear division is characterized, therefore, by the massing of the chromosomes from three of the megaspore nuclei on one large metaphase plate at the chalazal end of the megagametophyte. There is no actual fusion of chromatin material, but the chromosomes come together and then each divides in an equational division. The large metaphase plate would be expected to contain three times the haploid number, or approximately 54 chromosomes. The exact number would depend upon the distribution and possible loss of chromosomes in the two preceding meiotic divisions. Counts of this large metaphase plate have always been in the vicinity of 54, with a range of 51–57 chromosomes.

An anaphase stage of the division at the chalazal end in one megagametophyte gave a total of approximately 108 chromosomes between the two groups on the spindle. There is not necessarily an exact separation of 54 chromosomes to each pole. The separation is often unequal, and it is difficult to obtain exact counts of each anaphase group. As might be expected with such a large mass of chromosomes, there is some loss of chromatin matter from the nuclei, resulting in the appearance of relatively large micronuclei at completion of the division. At the micropylar end of the sac, where the remaining megaspore nucleus undergoes division, metaphase plates have given counts of 18 chromosomes, while others have had 21, 20, and 17. Lagging chromatin material was noted in only one instance (fig. 31), but no micronuclei have been observed in this division.

Thus, at the end of the third nuclear division, the megagametophyte is composed of two small nuclei at the micropylar end and two larger ones at the chalazal end. These larger ones may be arranged on the long axis of the megagametophyte (figs. 12, 34), or across it (fig. 10), depending upon the orientation of the spindle from which each arose. The large vacuole previously mentioned now completely separates the nuclei at the micropylar end from those at the chalazal end, and small vacuoles may appear elsewhere in the cytoplasm.

One megagametophyte was observed in which there were eight nuclei, all the same size; probably the fusion of three megaspore nuclei failed to take place. Instead, all four megaspore nuclei probably divided again, with the result that a megagametophyte of eight haploid nuclei was formed. The fourth and final division usually takes place at the chalazal end first. The two large nuclei first undergo division, followed by division of the two small nuclei at the micropylar end. It is not uncommon, however, for the final division to take place simultaneously in all the nuclei (figs. 13, 35).

When both nuclei participate, four large ones will result from the division, and



FIGS. 19-33.—Fig. 19, megaspore mother cell. Fig. 20, prophase I. Fig. 21, metaphase I. Fig. 22, chromosome bridge, meiosis I. Fig. 23, extruded chromatin, meiosis I. Fig. 24, two megaspore nuclei. Fig. 25, prophase II. Fig. 26, late anaphase II; figure incomplete. Fig. 27, four megaspore nuclei. Fig. 28, appearance of vacuoles. Fig. 29, polarization of megaspore nuclei. Fig. 30, metaphase, third nuclear division, micropylar and chalazal end; figure incomplete. Fig. 31, same, micropylar end; lagging chromatin at micropylar end. Fig. 32, anaphase, third nuclear division, chalazal end; figure incomplete. Fig. 33, telophase, third nuclear division, micropylar and chalazal end.  $\times 450$ .

these take different positions in the chalazal end (fig. 41). Often only one of the large nuclei will undergo complete division, while the other begins to disintegrate in the prophase stage (figs. 14, 37). When this occurs only two large nuclei result, while the disintegrating nucleus is still visible. The nucleus which disintegrates is generally the one at the extreme chalazal end. Here again micronuclei are sometimes evident, showing that some chromatin material was not included in the four nuclei.

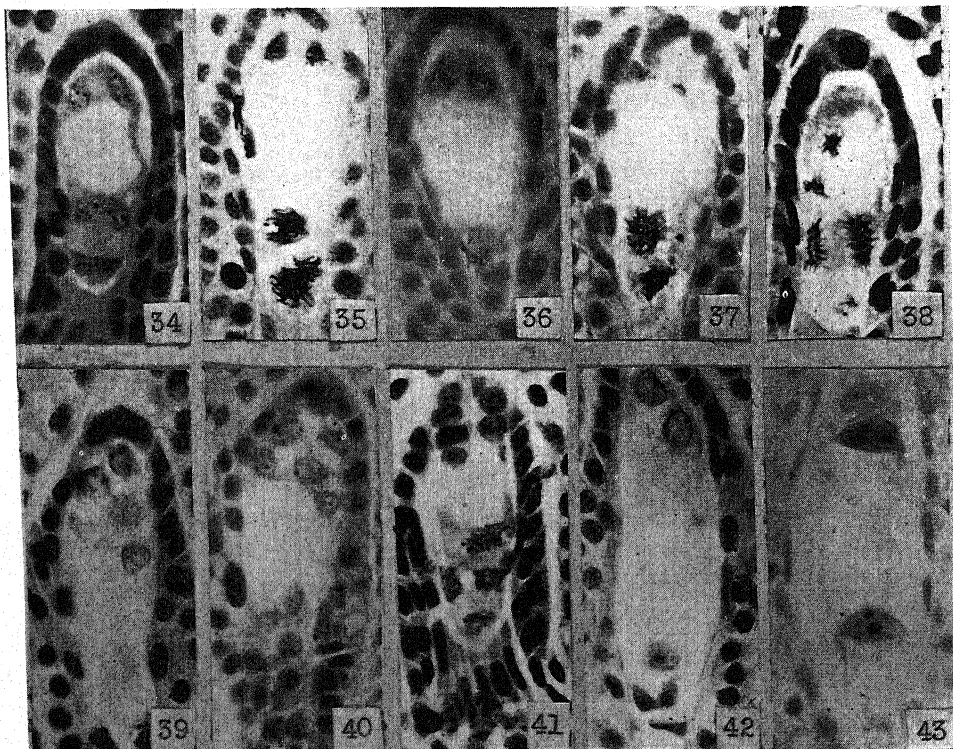
At the micropylar end, each of the two small nuclei undergo division, generally after the division is complete at the chalazal end. There seems to be no common arrangement of the spindles for this division, as they have been found oriented across the end of the megagametophyte and along either side (figs. 35, 36). In a few cases it was possible to determine the distribution of chromosomes in the anaphase stage. One had a 19-17 distribution for one nucleus and a 22-14 distribution for the other. Another showed a 20-16 distribution for one nucleus, but it was impossible to obtain an accurate count of the other. In one megagametophyte a total of 79 chromosomes was counted from the anaphase plates of the two dividing nuclei. This is an excess of 7 chromosomes over what would be expected had equal distribution taken place in the preceding divisions. The 79 chromosomes were so spread out in the cytoplasm that it was impossible positively to identify their respective nuclei. The four formed as a result of this division take different positions in the micropylar end (fig. 39).

In three rare cases five and six nuclei have been found at the micropylar end, indicating some form of irregularity in division. All the nuclei were approximately equal in size, and none appeared to be undergoing disintegration (fig. 40). The appearance of an occasional megagametophyte with five or six instead of four nuclei at the micropylar end may be brought about by failure of the scattered chromosomes to reconstitute themselves properly. No evidence of lagging or chromosome bridges was observed in this final division at the micropylar end.

At the completion of this division, the typical megagametophyte—allowing for irregular distribution and loss of chromosomes—is composed of four nuclei with approximately 18 chromosomes at the micropylar end, and two nuclei with approximately 54 chromosomes at the chalazal end. If both nuclei at the chalazal end of the sac take part in the final division, the total will be four instead of two large nuclei.

After four large nuclei have been formed at the chalazal end, the two at the extreme end first disintegrate, followed by the one next above (figs. 14, 15, 42). In some it appears that only the two at the extreme end disintegrate and the other two persist. The triploid nucleus nearest the center begins to increase in size and migrates toward the center of the megagametophyte (figs. 15, 42). One of the four small nuclei at the micropylar end also moves toward the center (figs. 16, 17).

Midway the large and the small nuclei fuse to form a polar apparatus of approximately 72 chromosomes. The three antipodals at the chalazal end are in various stages of disintegration (fig. 42); however, one of them may persist for some time. The three nuclei remaining at the micropylar end take up various positions, but one of them begins to increase in size about the time that fusion of the polar ap-



FIGS. 34-43.—Fig. 34, end of third nuclear division. Fig. 35, fourth nuclear division. Figs. 36, 37, metaphase, fourth nuclear division; note disintegrating chalazal nucleus. Fig. 38, anaphase, fourth nuclear division; figure incomplete. Fig. 39, end of fourth nuclear division, micropylar end; figure incomplete. Fig. 40, end of fourth nuclear division, micropylar end; note five nuclei; figure incomplete. Fig. 41, end of fourth nuclear division, chalazal end; figure incomplete. Fig. 42, megagametophyte showing three disintegrating antipodal nuclei, polar nucleus from chalazal end, polar nucleus at micropylar end and egg nucleus. Fig. 43, endosperm nucleus and egg nucleus.  $\times 450$ .

paratus is completed (fig. 43). Generally this is the nucleus nearest the center, and it becomes the egg cell. The two remaining synergids, and any extra nuclei that may have been formed through previous irregularities in division, begin to disintegrate, although one or more sometimes persist. Degeneration of the synergids and the antipodals presumably does not interfere with development of the egg and polar nuclei.

The typical megagametophyte, at the stage preceding fertilization, now consists of a large polar apparatus in the center and an egg nucleus at the micropylar end (fig. 43). The egg nucleus increases in size, while the polar nucleus appears to decrease considerably in the final stages of development. The megagametophyte is much vacuolated, especially between the nuclei and the surrounding nucellar layer. Finally a cell wall appears around each nucleus.

### Discussion

Formation of the *Fritillaria*-type (41) of megagametophyte has been somewhat incompletely described since the work of TREUB and MELLINK (43) in 1880 until COOPER's (14, 15) accounts in 1934 and 1935. The earlier workers (1, 2, 3, 4, 5, 10, 11, 18, 19, 21, 24, 31, 35) failed to present the complete and entire development. Since COOPER's work on *Lilium henryi*, numerous reports indicate that the *Fritillaria*-type is not confined to the Liliaceae (22, 28).

In *Tulipa*, BAMBACIONI and GIOMBINI (3) and BAMBACIONI-MEZZETTI (4) recognized certain early phases, but they were unable to follow the formation of the mature megagametophyte. BAMBACIONI-MEZZETTI (4) did state that in *T. gesneriana* the female gametophyte degenerated before it was mature and a type of megagametophyte originated by proliferation of the cells arising from the inner integument or the nucellus. NEWTON (33) stated that in the megagametophyte of the *Eriostemon*es group of *Tulipa* no fusion of megaspore nuclei took place at the chalazal end.

ROMANOV (36) has recently described two new forms in *Tulipa*, both of which differ from those described by NEWTON. Both forms are typical of their respective species and in both the development was analogous to the *Fritillaria*-type up to the third division. In neither species did the triple fusion of the three megaspore nuclei take place, so that in *T. rosea* a 14-nucleate gametophyte resulted and in *T. maximoviczii* a 5-nucleate one; yet four divisions were required to obtain the mature megagametophyte in each species. The single megagametophyte of Inglescombe Yellow, in which eight small nuclei were found, probably arose by failure of the three megaspore nuclei to fuse. Instead, all four nuclei underwent another division to give eight approximately haploid nuclei, a total of only three divisions. Had each of these undergone a fourth division a 16-nucleate gametophyte would have resulted, which would compare with the description given for *T. rosea* above. BAMBACIONI-MEZZETTI (5) reported one of the 8-nucleate type but did not consider its occurrence at all common.

Inglescombe Yellow closely follows the *Fritillaria*-type of development, and four divisions are necessary to form the mature gametophyte. However, although eight nuclei may be developed, the typical megagametophyte at the time of fertilization consists of only two. Degeneration of the two synergids and the three

antipodals, leaving the polar apparatus and the egg nucleus, is the common method of development.

The first division in the megagametophyte of Inglescombe Yellow is similar to that in the pollen mother cells with respect to the chromosome associations. Trivalents, bivalents, and univalents were found, but the number of trivalents seemed to be less than those reported for the male gametophyte by WOODS (48). It is possible that if a larger number of complete counts were available the number of trivalents would have been greater.

In subsequent divisions in the material observed, the occurrence of lagging chromosomes and of micronuclei was negligible. It seems, therefore, that the explanation for the predominance of  $3n \times 2n$  hybrids with high chromosome numbers (6) may be in part related to the comparative regularity of meiotic and subsequent divisions. This is in sharp contrast to the work of SATINA and BLAKESLEE (39) on *Datura*, where there was frequent loss of chromosomes by lagging from the female gametophyte. On the other hand, the results of their progeny studies were correspondingly different. UPCOTT and PHILP (46), from the chromosome numbers of  $3n \times 2n$  progeny of *Tulipa*, calculated that the female gametes should contribute close to 18 chromosomes, but they made no direct observations on the megagametophyte divisions. WESTFALL (47) examined the formation of the megagametophyte in *Lilium tigrinum* and found that the loss of lagging chromosomes in the meiotic and following divisions produced "a general shift in the distribution toward the lower chromosome numbers." He further determined that the distribution of chromosomes at the first meiotic division gives increases toward the  $1n$  and  $2n$  limits of distribution.

The frequent observations of unequal division of the number of chromosomes present in any one nucleus probably explains the occurrence of  $3n \times 2n$  hybrids with more or less than the mean of 30 chromosomes reported previously (6).

UPCOTT and PHILP (46) and SATINA, BLAKESLEE, and AVERY (40) have reported that the seeds resulting from  $3n \times 2n$  crosses are poorly developed. Our observations confirm this fact. The behavior of the chromosomes in the chalazal end of the embryo sac, where both unequal division after the triple fusion and to some extent lagging chromosomes occur, probably plays an important part in the viability of the seeds. It is not to be suggested that a loss of chromosomes or unbalance brought about by unequal distribution is directly responsible for the failure of these seeds to germinate, but rather that this unbalance affects the extent to which endosperm is produced, which in turn may affect the life of the seedling during germination.

### Summary

1. The megagametophyte of Inglescombe Yellow, a triploid tulip, is of the *Fritillaria*-type.



2. During the meiotic prophase of the first division trivalents, bivalents, and univalents were observed.
3. The occurrence of lagging chromosomes and micronuclei was infrequent.
4. There was unequal separation of the chromosomes in both meiotic and subsequent divisions.
5. The relation of these observations to triploid progeny is discussed.

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# EFFECT OF CONCENTRATION OF OXYGEN AND PRESSURE OF WATER UPON GROWTH OF RHIZOMES OF SEMI- SUBMERGED WATER PLANTS<sup>1</sup>

HARLOW E. LAING

(WITH SIX FIGURES)

During the course of a study of the respiration of the rhizomes of *Nuphar advenum* and other semi-submerged plants, it was observed that these organs were able to endure low oxygen tension and even complete absence of oxygen for a considerable period of time (7). It was also noticed that the presence or absence of oxygen affected their rate of growth. It was decided, therefore, to study the growth attained by the shoots under different conditions of aeration.

## Materials and methods

The plants used were *Nuphar advenum* Ait., *Typha latifolia* L., *Peltandra virginica* (L.) Kunth, *Pontederia cordata* L., *Acorus calamus* L., *Scirpus validus* Vahl, and *Sparganium eurycarpum* Engelm. Observations were also made on the land plants *Iris germanica* and *Ipomoea batatas*, for the sake of comparison.

The material was prepared so as to leave the buds or bases of the shoots exposed as much as possible without injury. If the petioles of a few of the large inner leaves of *N. advenum* were cut so as to leave about 3 cm. of the petioles surrounding the embryonic leaves, it was possible to control their further growth by adjusting the oxygen content of the medium. The mature rhizomes of *Typha latifolia* and *Sparganium eurycarpum* were prepared by carefully removing the terminal shoot and all the leaves cleanly to the rhizome, thus exposing the axillary buds but without injuring them. The petioles of *Peltandra virginica* and *Pontederia cordata* were cut squarely off. The fully developed membranous sheath, which remained stationary, made it possible to estimate the amount of new growth.

Except when otherwise stated, the rhizomes were put into suitable containers such as large test tubes or large-mouthed museum jars having rubber stoppers large enough to fit the tops and each fitted with two glass delivery tubes, and placed in a constant temperature bath kept at 25° C., which is about the temperature of the pond water of the native habitat during many of the warm days of summer. Moist gaseous mixtures containing various concentrations of nitrogen and oxygen from which the CO<sub>2</sub> had been removed were passed through the con-

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tainers steadily throughout each experiment, except when otherwise stated. The gases were mixed by means of a series of capillary tubes attached to manometers and governed by adjustable spill-bottles as previously described (7). The gases were drawn through the containers by a very slight negative pressure provided by an aspirator, and the pressure was kept constant by a mercury pressure regulator.

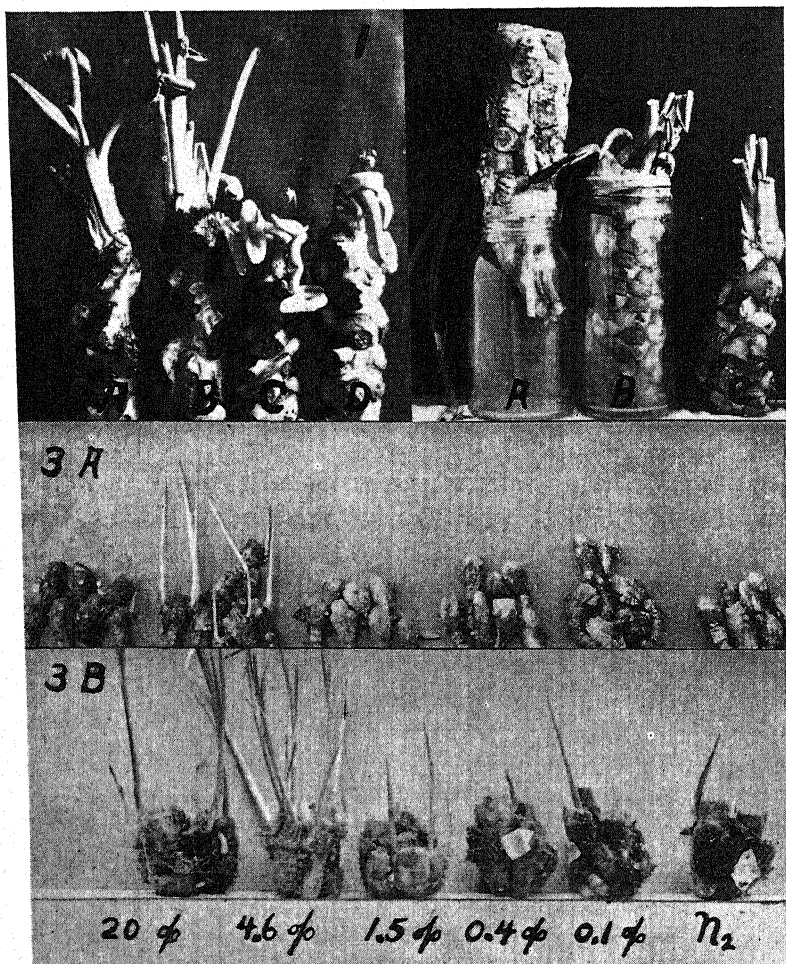
### Experimental results

#### EFFECT OF OXYGEN CONTENT OF MEDIUM ON GROWTH OF RHIZOMES

Two large rhizomes of *Nuphar advenum* were prepared as previously described, and each was submerged in water in a separate jar at room temperature. Air was bubbled through one jar and purified nitrogen through the other for 29 days. The results (fig. 1A, B) show that growth was approximately equal in both rhizomes.

To determine the effect of various concentrations of oxygen upon the growth of *N. advenum*, a number of rhizomes were prepared and subjected to concentrations of 20, 10, 4.6, 3, 1.5, 1, 0.4, and 0.1 per cent of oxygen by volume, and purified nitrogen, separately. Figure 4A shows that oxygen inhibits growth unless it is present only in very small quantities. The maximum rate of growth occurred in the rhizomes receiving from 1 to 0 per cent of oxygen. When the oxygen concentration exceeded 1.5 per cent, growth became very slow and ceased entirely at concentrations above 3 per cent. Leaves of *N. advenum* that had grown rapidly on rhizomes in nitrogen ceased elongating when the rhizomes were placed in air and became more stocky, the blades broadening slightly and the color changing from the previous pale etiolated condition to a glossy dark green. It was concluded that although anaerobic conditions are conducive to leaf growth, nevertheless when the leaves have once begun to unfold they are thereafter benefited by at least a small amount of oxygen. Apparently growth of *N. advenum* is not dependent upon an aqueous medium, but rather upon the fact that the aqueous medium limits the supply of oxygen.

Similar experiments were performed with the rhizomes of other species. *Peltandra virginica* and *Pontederia cordata* grew well in aerated water and in water through which nitrogen was passed, but *Typha latifolia* did not grow under either condition. When subjected to moist gaseous mixtures, *T. latifolia* grew best in an atmosphere containing 4.6 per cent of oxygen (fig. 3A), next best in 10 per cent, and third best in 3 per cent; no shoot growth occurred in concentrations of less than 3 per cent of oxygen and very little in 20 per cent of oxygen, that is, air. Although growth in this plant was inhibited in nitrogen and the lower concentrations of oxygen, nevertheless the buds themselves were able to endure a 13-day period of exposure to anaerobic conditions, as was shown by the following experi-



FIGS. 1-3.—Fig. 1, growth of rhizomes of *Nuphar advenum*: A, in aerated water; B, in water through which nitrogen was bubbled; C, under pressure of 2 feet of aerated water; D, under pressure of 4 feet of same. Fig. 2, growth of rhizomes of *N. advenum*: A, with apex in air and remainder in nitrogen; B, with apex in nitrogen and remainder in air; C, control, entirely surrounded by nitrogen. Growth occurs when apex is in nitrogen and not when it is in air, regardless of which gas surrounds remainder of rhizome. Fig. 3, growth of rhizomes of *Typha latifolia*: A, after 13 days in air, in various concentrations of oxygen, and in nitrogen; B, same rhizomes 21 days later, after having been exposed to slow stream of moist air in bell jar for 21 days.

ment. After being photographed, the rhizomes shown in figure 3A were carefully tied together in little bundles, each group by itself. These bundles were put into a bell jar through which moist air was passed very slowly for 21 days, at the end of which time they were photographed again while still tied together (fig. 3B). The subsequent growth shows that many of the buds had survived the previous lack of oxygen.

In contrast to *Typha latifolia*, both *Peltandra virginica* and *Pontederia cordata* grew well in nitrogen and the lower concentrations of oxygen (fig. 4B, C), but *P. virginica* did not grow perceptibly in concentrations of oxygen as high as 10 per cent; and although *P. cordata* grew well in 10 per cent of oxygen, it too was somewhat retarded in air.

Rhizomes of *Acorus calamus*, *Sparganium eurycarpum*, and *Scirpus validus* were exposed for 8 days to 20, 10, 3, 1.5, and 1 per cent of oxygen and nitrogen. The results are given in table 1. The fact that the rhizomes of *A. calamus* produced in low oxygen concentrations shoots that were able to live and grow through the experiment showed that these rhizomes were able to endure a considerable period of anaerobiosis. But they did not grow rapidly unless they had more than 3 per cent and somewhat less than 20 per cent of oxygen. Best growth was at 10 per cent, but probably the optimum concentration is either a little above or a little below this percentage. In *S. eurycarpum* the shoots were not only able to endure anaerobic conditions but actually grew as well in 1.5 per cent of oxygen as in moist air.

In *Scirpus validus* growth was definitely better at 10 per cent of oxygen than at the other concentrations. Growth in the lower concentrations and in nitrogen undoubtedly began before the original air of the containers had all been displaced by the incoming gases. Although it is not known how long this growth was sustained, it was certain at the end of the experiment that all the shoots had died in the jars containing only 1 per cent of oxygen and in nitrogen, and were dying in 1.5 per cent of oxygen. In another experiment, not reported here, it was found that the buds on rhizomes of *S. validus* that had remained dormant in nitrogen continuously for 8 days without injury were able to grow when placed in moist air, and that shoots that had previously been formed in moist air were killed in nitrogen.

Throughout all these experiments, observations were made of any noticeable root growth. In both *Typha latifolia* and *Pontederia cordata* the best root growth occurred in air, the next best in 10 per cent, next in 4.6 per cent of oxygen, etc.; but no root growth occurred in concentrations of less than 1.5 per cent of oxygen. In *T. latifolia* root growth practically ceased in 1.5 per cent. Toward the end of the experiment with *P. cordata* the nitrogen and air tubes were exchanged, so

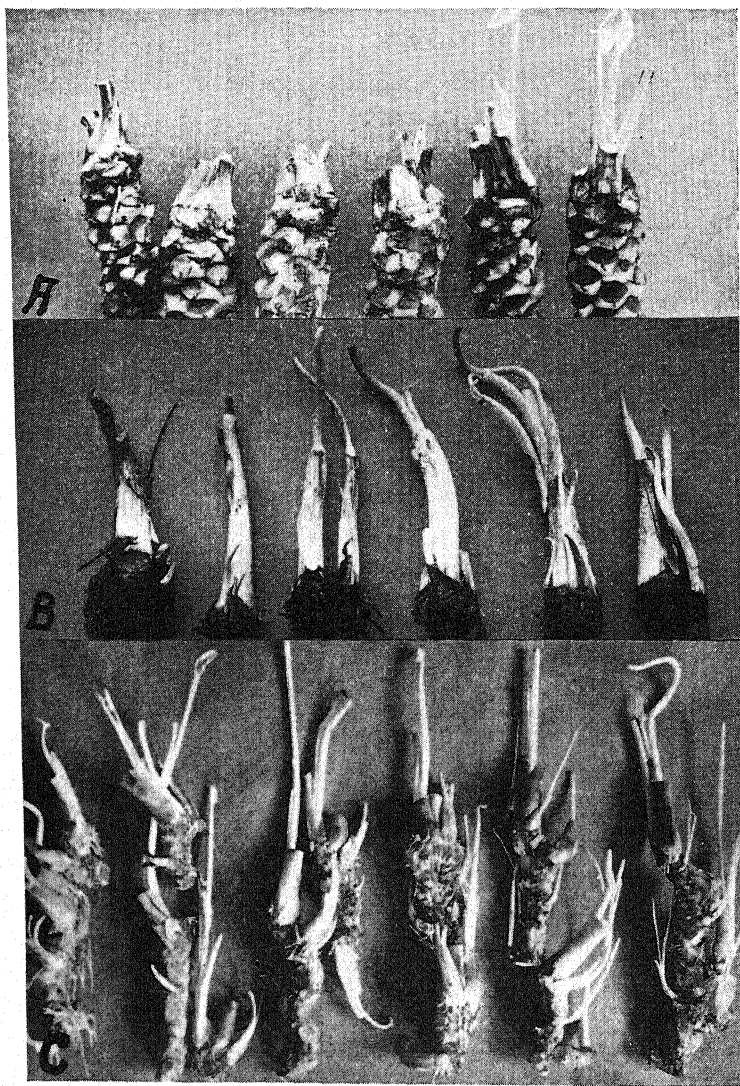


FIG. 4.—Growth of rhizomes in (reading from left to right) air, 10%, 3%, 1.5%, 1% of oxygen, and in nitrogen: A, *Nuphar advenum* after 11 days; B, *Peltandra virginica* after 8 days; C, *Pontederia cordata* after 11 days.

that the samples that had been in air for 8 days were continued in nitrogen for 3 days, and those that had been in nitrogen for 8 days were continued in air for 3 days. This exchange was sufficient to induce a beginning of root formation on the rhizome that had been in nitrogen previously (fig. 4C, N<sub>2</sub>). On the other hand, the roots previously formed in air were injured by the nitrogen, and many of them were dying at the close of the experiment. This experiment shows that at least some of the root primordia of *P. cordata* are not injured by nitrogen, but that they do not begin to grow until there is a sufficient supply of oxygen. After growth has progressed to the extent that the roots have extended into the medium, the

TABLE 1

MAXIMUM LENGTH OF GROWTH, IN MILLIMETERS, OF SHOOTS AND ROOTS OF ACORUS CALAMUS, SPARGANIUM EURYCARPUM, AND SCIRPUS VALIDUS DURING 8 DAYS IN VARIOUS CONCENTRATIONS OF OXYGEN

SPECIES	20% O <sub>2</sub>	10% O <sub>2</sub>	3% O <sub>2</sub>	1.5% O <sub>2</sub>	1% O <sub>2</sub>	N <sub>2</sub>
<i>A. calamus</i> .....	65	80	45	30	25	10
<i>S. eurycarpum</i> .....	25	7	15	25	8	5
<i>S. validus</i> .....	70	120	45	30 (dying)	40 (dead)	90 (dead)

roots thereafter are very sensitive to a lack of oxygen, or at least are harmed by nitrogen from which oxygen and CO<sub>2</sub> have been removed.

Included with the preceding experiments were some rhizomes of *Iris germanica* and some tuberous roots of *Ipomoea batatas*. The leaves of *Iris* were cut off uniformly, leaving about 3 cm. at the base. Some young shoots 1–2 cm. in length having small expanding leaves were present on *Ipomoea* at the beginning of the experiment. The results showed that the leaves of *Iris* cannot endure the conditions of oxygenation below 3 per cent. The only growth that occurred was in air and in 10 per cent of oxygen. The leaves grew nearly as well in 10 as in 20 per cent of oxygen, but the roots grew best in 20 per cent. The leaves that had already grown on *Ipomoea* previous to the experiment were able to endure anaerobiosis to some extent but were injured at concentrations below 1.5 per cent. Root growth was good in air and in 10 per cent of oxygen.

Experiments were performed on various other species of semi-submerged water plants and also on species of land plants. The results were not different from those already obtained.

In summarizing this group of experiments it may be said that the growth of semi-submerged plants is hindered by fully aerobic conditions, but that some oxygen is favorable to the growth of certain species, the optimum being different for different species. Also the optimum range of oxygen concentration is comparatively broad for some species, such as *Pontederia cordata*, but is relatively narrow



for others, such as *Typha latifolia*, *Peltandra virginica*, and *Nuphar advenum*. One striking result is the fact that the initiation of growth in some species required no oxygen whatever, and that in *N. advenum* oxygen was detrimental to growth when present in amounts exceeding 1 per cent.

#### EFFECT OF OXYGEN CONTENT OF INTERNAL ATMOSPHERE ON GROWTH OF NUPHAR ADVENUM

To determine whether or not the oxygen content of the internal atmosphere of the rhizome of *N. advenum* exerts an effect on the growth of the leaves, the following two experiments were performed.

In the first experiment analyses were made of the internal atmospheres of a number of rhizomes under different conditions. The gases were extracted at room temperature by means of an apparatus previously described (9) and analyzed in a Henderson-Haldane apparatus. The internal atmosphere of a rhizome kept in a stream of nitrogen for 10 days contained 2 per cent of CO<sub>2</sub> and no oxygen; that of a rhizome kept in CO<sub>2</sub> for 11 days contained 75 per cent CO<sub>2</sub> and no oxygen; that of another rhizome kept in stale water saturated with CO<sub>2</sub> under a slight excess pressure contained 88 per cent of CO<sub>2</sub> and no oxygen; while that of a rhizome lying on the laboratory table under a moist cloth contained 19.6 per cent of oxygen and only 1.2 per cent of CO<sub>2</sub>. The air of the laboratory contained 20 per cent of oxygen. These results indicate that the composition of the internal atmosphere of a detached leafless rhizome of this plant tends to resemble that of the gaseous medium, allowing for certain slight differences due undoubtedly to respiration or fermentation.

In the second experiment two uniform rhizomes were placed in fruit jars in such a manner that only the leaves of the bud of one of the rhizomes extended above the jar (fig. 2*B*), while only the leaves of the bud of the other rhizome protruded into the other jar and the remainder of the rhizome extended above it (fig. 2*A*). The jars were made air-tight by sealing the rhizomes in with plastel-line. Tubes were inserted so that moist air could be passed through each jar. The jars were then placed in a large bell jar, and the tubes were connected through a large rubber stopper so that air could be circulated through the small fruit jars, while nitrogen was being circulated through the remainder of the bell jar. An extra rhizome was placed in the bell jar to serve as a control. The results of this experiment are shown in figure 2. The terminal bud that extended into the nitrogen-filled bell jar grew, regardless of the fact that the remainder of the rhizome undoubtedly contained much oxygen since it was surrounded by an atmosphere containing 20 per cent inside the small jar (fig. 2*B*). The control in the atmosphere of nitrogen grew also (fig. 2*C*). But the rhizome, the bud of which protruded into the air-filled fruit jar, did not grow although the remainder of its bulk un-

doubtedly contained little or no oxygen since it extended into the nitrogen-filled bell jar (fig. 34).

These two experiments show that the anaerobic conditions that induce growth must be applied to the exterior of the bud, and that growth of the shoot is not noticeably affected by the oxygen concentration of the internal atmosphere of the rhizome.

#### EFFECT OF PRESSURE ON GROWTH OF RHIZOMES OF NUPHAR ADVENUM

*Nuphar advenum* is rarely, if ever, found growing in water exceeding 6-7 feet in depth. Experiments were conducted, therefore, to determine whether pressure is a limiting factor on growth.

In the first experiment a large vigorous rhizome that had grown well in 0.1 per cent of oxygen and a recently dug, short vigorous rhizome that had been kept under moist towels for 6 days—so that aerobic dormancy was fully established—were both put into a jar and connected so that they received nitrogen gas under a reduced pressure equal to 30 inches of water less than atmospheric pressure. Five days later another short, freshly dug rhizome was put into the jar with the other two.

On the eighteenth day of the experiment no additional growth had occurred on the large rhizome that had grown previously in 0.1 per cent of oxygen, and the leaves previously produced were dying. They were still pallid, showing that no air had entered the jar. The short rhizome that was put in at the beginning of the experiment had barely begun to grow. The short rhizome that had been put in on the fifth day had not grown. It was therefore decided to increase the pressure of nitrogen to an amount equal to 24 inches of water more than atmospheric pressure. This was accomplished by the device shown in figure 5. The nitrogen gas, after passing through the respiration jar, was compelled to displace 24 inches of water before escaping in bubbles.

Within 12 days the two short rhizomes produced a growth of 30 mm. and 50 mm., respectively, showing that the previously reduced pressure of nitrogen, while hindering growth, had not destroyed the tissues. The larger rhizome failed to recover.

The next experiment employed a variety of pressures. Two rhizomes were put into each of five jars, and these were arranged so that the amount of water being displaced by the escaping nitrogen was 2, 4, 6, 8, and 10 feet respectively. Growth occurred in all the rhizomes; the average amount in the lower pressures exceeded that in the higher.

To determine how long these pressures of nitrogen could be endured, the rhizomes were returned to the jars. This time, however, one or both from each jar

were exchanged for a rhizome from another jar or for a fresh one in order to note any differences in growth. Growth occurred under all pressures, even as high as the equivalent of 10 feet of water more than atmospheric pressure, but previously formed leaves did not endure such high pressure very long. The most suitable pressures were 2, 4, and 6 feet.

This experiment was repeated in a medium of aerated water instead of nitrogen gas, by means of the device shown in figure 6. The continuous column of

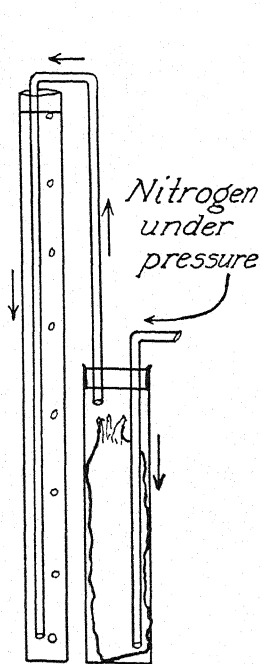


FIG. 5

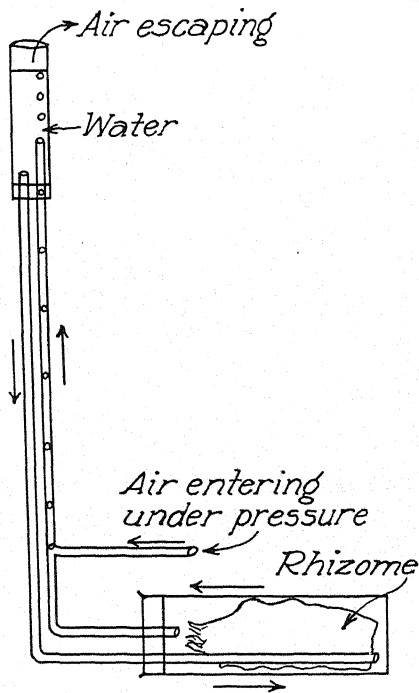


FIG. 6

FIGS. 5, 6.—Fig. 5 (left), device used to produce excess pressure of nitrogen equal to given number of feet of water pressure. Fig. 6 (right), device used to produce effective controlled pressure of given number of feet of aerated water.

water, circulating through the two tubes opening into an elevated reservoir above and into the respiratory jar lying horizontally below, was kept in motion and aerated by air under pressure which, rising in bubbles, forced the column of water to move as indicated by the arrows. Dye was introduced to make certain that the water was actually circulating. The desired pressure was obtained by adjusting the distance of elevation between the reservoir and the respiratory jar. The pressures used were 2, 4, 6, 8, and  $9\frac{1}{2}$  feet of water. Best growth occurred in 2 and 4 feet, but growth was very satisfactory at the higher pressures.

The results of another experiment conducted similarly are shown in figure 1C, D. The rhizomes C and D made excellent growth of leaves in 2 and 4 feet of water, respectively, during 18 days. The contortion of the petioles is due to their cramped position in the closed horizontal jars. There was no noticeable difference in the average daily rate of growth of these two rhizomes and the two previously described that were not subjected to pressure (fig. 1A, B).

These experiments show that pressure up to  $9\frac{1}{2}$  feet of water does not prevent growth, but that growth is better under pressure not exceeding 4-6 feet.

### Discussion

From these experiments it may be concluded that the adaptation of the different species of semi-submerged water plants to various depths of water is at least partly due to the different oxygen requirements for maximum growth of the young shoots. Although *Typha latifolia*, *Sparganium eurycarpum*, *Scirpus validus*, and *Acorus calamus* were able to respire anaerobically and to endure anaerobic conditions in the dormant state, nevertheless they required appreciable amounts of oxygen to sustain growth and respiration in the active state. This is associated with the observation that these plants normally flourish in comparatively shallow water and are also to be found growing on well-aerated muddy shores that are inundated only part of the season. In fact, the manner of growth of *T. latifolia*, *S. eurycarpum*, and *A. calamus* is such that usually some of the shoots are in contact with air the year round. In winter, when the old leaves have died, young green shoots may be found pressed in between the old leaves and extending above the ice. In the latitude of Chicago and Detroit, these shoots stay green all winter and the more vigorous ones give rise to fruiting culms the following summer. It is undoubtedly this need of oxygen that prevents their successful invasion of deep water. It must not be forgotten, however, that growth was found to be retarded by too much oxygen as well as by too little. This is associated with the observation that these plants, while able to grow for some time on well-aerated muddy shores, nevertheless do not succeed so well there as in shallow marshy water, and, unless inundation occurs occasionally, the colonies eventually disappear.

The retardation of growth in air of the young shoots of *Nuphar advenum*, *Peltandra virginica*, and *Pontederia cordata* was even more pronounced than in the previously mentioned species. This is associated with the observation that these plants normally grow best where the water is at least 12 inches deep, and that growth becomes promptly retarded and soon ceases altogether on well-aerated muddy shores left by the subsidence of the water level. *N. advenum* seems to be affected more quickly than the others. This agrees with the fact that it is the most sensitive to oxygen. A somewhat similar condition was found in *Limnanthemum nymphaeoides* by FUNKE (5), who reported that the young leaves grew best

when the oxygen content of the water was restricted and poorest when the water was well aerated. This injury to growth by too much oxygen is not confined merely to water plants. In certain unpublished studies, the writer found that buds of *Aesculus hippocastanum* and various other trees and shrubs from which the scales had been carefully removed grew readily in an atmosphere containing 10 per cent of oxygen but were greatly retarded in air. KIDD (6) and THORNTON (11) found that the thin-skinned freshly harvested tubers of potatoes sprouted readily in atmospheres containing 2-10 per cent of oxygen, but not in air. WENT and THIMANN (13) stated that growth hormones are easily oxidized and rendered functionless, but whether or not that accounts for the retarding effect of oxygen on growth is uncertain.

Although the young leaves of *Nuphar advenum* grew as rapidly in nitrogen as in 1 per cent of oxygen, those growing in nitrogen remained etiolated until oxygen was introduced, but growth was retarded when the oxygen concentration exceeded 1 per cent. As is pointed out elsewhere (8), mature aerial leaves of *N. advenum* succumbed to the lethal effects of anaerobic conditions about as soon as did those of *Pelargonium zonale*. The exact nature of the change of adaptation of these leaves from anaerobic to aerobic conditions is unknown.

A difference in respect to the resistance of leaves of different species to anaerobic conditions was also observed. Similar observations have been made by DEHERAIN and MOISSAN (3) and their practical importance pointed out by SCURTI and ZAVANAJU (10).

Although root growth was not studied primarily, it was observed that the newly formed roots of *Pontederia cordata* were killed when subjected to an atmosphere of pure nitrogen, and that, regardless of the species, the best root growth invariably occurred in concentrations of 10 or 20 per cent of oxygen. Similar observations have been made by other investigators (2, 12, 4, 1). It should be pointed out that although roots grow well in moist air, that in itself is no reason for supposing that such high concentrations of oxygen are essential for root growth. Just why the young roots of such a species as *Typha latifolia* should thrive in moist air while the young shoots are markedly retarded is unknown.

Another significant point brought out in these experiments is the fact that the anaerobic conditions that induce growth in *Nuphar advenum* must be applied to the exterior of the bud, and that the growth of the shoot is not noticeably affected by the oxygen concentration of the internal atmosphere of the rhizome. This fact is of distinct physiological and ecological interest because, as has been shown by other experiments (9), during the growing season the internal atmosphere of the apex of the rhizome—which at night may contain only a very small amount of oxygen—in the daytime may contain as much as 10 per cent, obtained chiefly as a by-product of photosynthesis. This internal oxygen is respired within the rhi-

zome, and little if any can enter the very young leaf because of the numerous dense layers of undifferentiated tissue that compose the abscission zone at this stage. As the petiole grows the air cavities become enlarged and more extensive, until finally air passages are opened up through the abscission zone and gases pass freely between the rhizome and the petiole.

Besides the oxygen tension, it was also found that the pressure exerted upon the rhizomes slightly influenced the development of new shoots. A reduced pressure equal to 30 inches of water less than atmospheric pressure inhibited growth of the young leaves of *N. advenum* in an atmosphere of moist nitrogen. The young leaves, however, grew in the atmosphere of moist nitrogen under positive pressure equal to as much as 10 feet of water. The average growth, however, was better at 2, 4, and 6 feet than at 8 and 10 feet. Similarly in aerated and non-aerated stale water, pressure equal to 9½ feet did not prevent growth, but growth was best at 2, 4, and 6 feet. It is concluded that water pressure, in itself, is not the factor that prevents the migration of the colonies into deeper water.

### Summary

1. Semi-submerged water plants inhabit their particular habitats very largely on the basis of adaptation in respect to their oxygen requirements for respiration and growth. The oxygen requirements for growth are different for different species.

2. The percentage of oxygen found to be most conducive to the growth of young shoots in certain of the species studied was, respectively, *Nuphar advenum* 0 to 1, *Peltandra virginica* 0 to 1.5, *Pontederia cordata* 0 to 10, *Typha latifolia* 4.6, *Acorus calamus* 10, *Scirpus validus* 10. Buds of all these species were able to endure prolonged exposure to pure nitrogen, but no growth occurred in *A. calamus*, *S. validus*, and *T. latifolia* in nitrogen. Shoots of *S. validus* previously formed were killed in nitrogen.

3. The excellent growth of *N. advenum* in nitrogen was not affected by either the presence or absence of oxygen in the internal atmosphere of the rhizome so long as the apex was surrounded by nitrogen. When the apex was surrounded by air, no growth occurred even though the rest of the rhizome was surrounded by nitrogen.

4. Even though the shoots of most of the water plants studied grew best in a medium having a low oxygen content and rarely in one having a high content, nevertheless the roots invariably grew best when the oxygen content was high and never when it was very low. Roots previously formed in air were killed in nitrogen.

5. Pressure of water as high as 9½ feet did not prevent growth in *Nuphar advenum*, but growth was usually better under pressures not exceeding 4-6 feet.

A pressure of nitrogen gas equivalent to 10 feet of water did not prevent growth, but the young leaves were unable to endure this high pressure for as long as they could endure pressures equivalent to 2 and 4 feet. Growth did not occur in a reduced pressure of nitrogen gas equal to 30 inches of water less than atmospheric pressure.

This investigation was conducted under the direction of Dr. F. G. GUSTAFSON, to whom the writer is indebted for various suggestions and encouragement. He also wishes to thank Drs. C. D. LA RUE and C. C. MELOCHE for helpful information. During part of the time that this research was conducted, the writer was the holder of an F. C. and Susan Eastman Newcombe fellowship in plant physiology.

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# PHASIC DEVELOPMENT OF MARQUIS SPRING WHEAT AND FULHIO WINTER WHEAT

D. J. WORT

(WITH TWO FIGURES)

## Introduction

The theory of phasic development of plants assumes that progress toward reproductive maturity consists of a series of phases occurring in strict sequence. The environment favorable for completion of one phase may be altogether unsuitable for the initiation and progress of the subsequent phase. No matter whether conditions are favorable for the completion of later phases, the plant cannot advance toward reproductive maturity unless the previous stage has been completed.

An analysis of the literature suggests the existence of at least four phases, which in order are: (1) prethermo-phase or phases, (2) thermo-phase, (3) scoto- or dark-phase, and (4) photo-phase. The photo-phase may consist of a flowering phase and a gametogenesis phase. Of the four, the thermo- and photo-phase have received most emphasis.

The experiments described here were designed to investigate (1) the thermo-phase of Marquis spring wheat in relation to its total or partial completion during seed ripening, and the effect of this completion on the response of the ripened seed to vernalization treatment; (2) the scoto-phase of Marquis wheat and Fulhio winter wheat, and the possibility of its completion under an 8-hour photoperiod; and (3) whether any portion of the photo-phase of these wheats could be passed as quickly under an 8-hour photoperiod as under continuous light.

## Methods

The procedure for vernalization and criteria for heading and flowering are those already outlined (16). The vernalization formulas are indicated in each section. Controls received the same amount of water as the treated seeds and were then allowed to germinate for 24 hours at 20° C.

Five plants were grown in 4½-inch pots filled with well-mixed loam soil. When an 8-hour photoperiod was used the plants were placed in complete darkness for 16 hours out of each 24-hour period by covering with black cloth, or trucks holding the pots were run into lightproof compartments. Sixteen-hour photoperiods (16 hours of light followed by 8 hours of darkness) and 24-hour photoperiods (con-



tinuous light) were obtained by supplementing the natural day length with light from 200-watt Mazda filament lamps suspended 24 inches above the tops of the pots.

Vernalization treatments were begun at such times so that all were concluded on the same day, February 24, 1940, for the original experiments and April 12, 1940, for the repetition of the experiments.

#### THERMO-PHASE OF MARQUIS WHEAT

To obviate the possibility of natural vernalization of seed during ripening (4, 6, 17), Marquis spring wheat was grown in the greenhouse during the autumn of 1939. The temperature in the greenhouse did not fall below  $21.1^{\circ}\text{C}$ . ( $70^{\circ}\text{F}$ .) at any time. At the soft dough stage a number of pots were placed in a coldroom, the temperature of which was  $1.9 \pm 0.9^{\circ}\text{C}$ . The plants were not illuminated. Pots of wheat were removed from the cold chamber at the end of 2, 4, 6, 8, and 10 days and returned to the greenhouse to ripen.

These seeds, together with seeds of plants which had not experienced vernalization temperatures during growth, were vernalized for 0, 4, and 8 days at  $3^{\circ}\text{C}$ ., the water added being 60 per cent of the air-dry weight. Seven pots of five plants each were grown for each combination of treatments, a total of 630 plants in all. These were grown in a greenhouse, the minimum temperature of which was  $21.1^{\circ}\text{C}$ ., under a photoperiod of 16 hours:

#### SCOTO-PHASE OF MARQUIS SPRING WHEAT AND FULHIO WINTER WHEAT

The seed of Marquis spring wheat, the ripening conditions of which were unknown, was vernalized using the formula 8:3:60 (8 days chilling at  $3^{\circ}\text{C}$ ., the water added being 60 per cent of the air-dry weight of seed), and Fulhio winter wheat using the formula 36:3:40.

Forty-two pots of vernalized and forty-two pots of unvernallized Marquis wheat were placed in a darkroom, the temperature of which was  $21.1^{\circ}\text{C}$ . or above, and at the end of 4, 8, and 12 days following emergence removals of pots were made to a 16-hour and to a 24-hour photoperiod. Likewise fifty-six pots of both vernalized and unvernallized Fulhio winter wheat were placed in the darkroom; and at the end of 5, 10, 15, and 20 days following emergence, removals of pots were made to a 16-hour and to a 24-hour photoperiod. Each removal involved seven pots of vernalized and seven pots of unvernallized wheat.

Seventy-seven pots of vernalized and seventy-seven pots of unvernallized Marquis and 133 pots of vernalized and 133 pots of unvernallized Fulhio wheat were placed on the 8-hour bench. Removals to a 24-hour photoperiod were made at intervals of 5 days following emergence. This continued for 40 days (eight trans-

fers) in the case of Marquis and for 75 days (fifteen transfers) in the case of Fulhio wheat. Similar transfers were made to a 16-hour photoperiod, the intervals being 5, 10, and 15 days following emergence in the case of Marquis and 5, 10, 15, and 20 days in the case of Fulhio wheat.

In a repetition of the experiment (experiment B), Marquis wheat, vernalized and unvernallized, was sown on April 12, 1940. Transfers from darkness to continuous light were made at the end of 0, 4, 7, and 10 days following emergence. No 16-hour photoperiod was used.

#### PHOTO-PHASE OF MARQUIS AND FULHIO WHEATS

The environment to which the seeds used in this section were exposed during ripening is not known. The formula used in vernalizing the Marquis wheat was 8:3:60; for the Fulhio wheat, 36:3:40. The plants were grown on a 24-hour photoperiod. Five days after emergence, seven pots of unvernallized and seven pots of vernalized Marquis and Fulhio wheat were removed from the 24-hour to an 8-hour photoperiod. Removals were made every 5 days thereafter until 35 days had elapsed in the case of Marquis and 75 days in the case of Fulhio. In a repetition of the experiment, Marquis wheat was sown on April 12. Removals were made 4 days after emergence and every 3 days thereafter for a total of 28 days, thus involving 700 plants.

### Results

#### THERMO-PHASE OF MARQUIS SPRING WHEAT

The results of the investigation of the thermo-phase of Marquis spring wheat are given in table 1. The average number of days to head and flower are given to the nearest whole number. The seeds which were not vernalized and which were obtained from parent plants which had not experienced temperatures below 21.1° C. (and hence presumably had not been vernalized) were considered the controls. Flowering of plants grown from control seed occurred 57 days after planting.

Vernalization of the control seed for 4 and 8 days produced an acceleration in flowering of 6 and 4 days, respectively. Treatment of the plants with cold for 2, 4, 6, 8, and 10 days when the grain was ripening accelerated flowering of plants subsequently grown from the seed by 1-2 days in all cases except one. Apparently the acceleration caused by vernalization of seed from chilled plants is less than that from unchilled.

To eliminate as far as possible the role of continuous darkness and constant low temperature during chilling of the ripening seed, Marquis spring wheat was grown in the field during the summer and autumn of 1939, and early-ripening and

late-ripening plants harvested separately. The air and soil temperatures for the entire growing season were recorded by thermographs.

Flowering and heading times of the 300 plants grown in the greenhouse from the seed collected in the field are shown in table 2. The early-ripened seed, which experienced little if any chilling during ripening, responded to vernalization treat-

TABLE 1  
EFFECT ON HEADING AND FLOWERING OF PLANTS OF MARQUIS SPRING WHEAT  
GROWN FROM SEED CHILLED DURING RIPENING AND VERNALIZED  
PRIOR TO PLANTING

NO. OF DAYS PLANT CHILLED DURING SEED RIPENING	NO. OF DAYS SEED VERNAL- IZED PRIOR TO PLANT- ING	AVERAGE NO. OF DAYS TO HEAD	AVERAGE NO. OF DAYS TO FLOWER	ACCELER- ATION OF FLOWER- ING DUE TO CHILL- ING OF PLANT	ACCELER- ATION OF FLOWER- ING DUE TO VERNALIZA- TION OF SEED	TOTAL ACCELER- ATION OF FLOWER- ING	NO. OF PLANTS
0.....	0	53	57	.....	.....	.....	35
2.....	0	50	55	2	.....	2	35
4.....	0	54	55	2	.....	2	35
6.....	0	56	57	0	.....	0	35
8.....	0	54	56	1	.....	1	35
10.....	0	55	56	1	.....	1	35
0.....	4	50	51	.....	6	6	35
2.....	4	50	51	2	4	6	35
4.....	4	52	52	2	3	5	35
6.....	4	52	53	0	4	4	35
8.....	4	54	55	1	1	2	35
10.....	4	49	51	1	5	6	35
0.....	8	51	53	.....	4	4	35
2.....	8	50	53	2	2	4	35
4.....	8	54	55	2	0	2	35
6.....	8	53	54	0	3	3	35
8.....	8	54	55	1	1	2	35
10.....	8	54	55	1	1	2	35
Total plants.....							630

ment for 4 and 8 days with accelerations of flowering of 4 and 6 days, respectively. The late-ripening grain, which had experienced air temperatures of 10° C. or below during 8 days and soil temperatures of 10° C. or below during 21 days, was less sensitive to treatment. The accelerations of flowering were 2 and 3 days, respectively.

The claim of KOSTJUCENKO and ZARUBAILO (6), GREGORY and PURVIS (4), and others that vernalization can occur during seed ripening is substantiated by these laboratory and field results. That the thermo-phase was not entirely com-

pleted during ripening, under the conditions of these experiments, was evident from the fact that a positive response to vernalization of the seed was obtained.

### SCOTO-PHASE OF MARQUIS AND FULHIO WHEATS

Exposure of unvernallized and vernalized Marquis spring wheat to darkness for 4-12 days after emergence from the soil and then transferring to a 24-hour photoperiod did not decrease the total time required for flowering. The results of experiment A (planted February 24), involving 1190 plants, and of experiment B

TABLE 2  
HEADING AND FLOWERING DATA OF PLANTS GROWN FROM  
VERNALIZED AND UNVERNALLIZED MARQUIS WHEAT  
SEED PRODUCED IN THE FIELD

WHEN RIPENED IN FIELD	DAYS SEED VERNALL- IZED PRIOR TO PLANT- ING	AVERAGE NO. OF DAYS TO HEAD	AVERAGE NO. OF DAYS TO FLOWER	ACCELE- RATION OF FLOWERING DUE TO VERNALLIZA- TION OF SEED	NO. OF PLANTS
Early.....	0	53	54	.....	50
Early.....	4	48	50	4	50
Early.....	8	47	48	6	50
Late.....	0	53	53	.....	50
Late.....	4	49	51	2	50
Late.....	8	48	50	3	50
Total plants.....					300

(planted April 12), involving 490 plants, are given in table 3. The results of experiment A are shown graphically in figure 1. Thirty-five vernalized and thirty-five unvernallized plants were grown at each treatment. Only the data for unvernallized plants are included, as vernalization caused no constant acceleration of flowering. The heading and flowering times are the average for the first twenty plants to head and to flower.

Irrespective of the length of exposure to darkness, all plants required approximately the same number of 24-hour photoperiods to flower: forty in experiment A, thirty-eight in experiment B. The total time from emergence to flowering was increased by the length of exposure to darkness.

When an 8-hour photoperiod was substituted for darkness, the shorter exposures had little effect on the number of 24-hour photoperiods subsequently required to produce flowering of Marquis wheat. Longer exposure to an 8-hour photoperiod reduced the number of 24-hour photoperiods required by two to five (table 3).

As in the case of exposure to darkness, the total number of days from emergence to flowering was increased by the 8-hour treatment. Results similar to those involving a 24-hour photoperiod were obtained when darkness or an 8-hour photoperiod was followed by a 16-hour photoperiod (table 3).

TABLE 3  
EFFECTS OF DARKNESS AND 8-HOUR PHOTOPERIOD FOLLOWING EMERGENCE ON  
TIME TO HEAD AND TO FLOWER BY MARQUIS SPRING WHEAT AND  
FULHIO WINTER WHEAT

EX- PERI- MENT	DAYS OF DARKNESS AFTER EMERGENCE	ADDITIONAL 24-HOUR DAYS		To- TAL DAYS TO FLOW- ER	ADDITIONAL 16-HOUR DAYS		To- TAL DAYS TO FLOW- ER	No. OF 8-HOUR DAYS AFTER EMERGENCE	ADDITIONAL 24-HOUR DAYS		To- TAL DAYS TO FLOW- ER	ADDITIONAL 16-HOUR DAYS		To- TAL DAYS TO FLOW- ER	
		To HEAD	To FLOW- ER		To HEAD	To FLOW- ER			To HEAD	To FLOW- ER		To HEAD	To FLOW- ER		
MARQUIS SPRING WHEAT															
A	{	0.....	35	40	40	52	54	54	0.....	35	40	40	52	54	54
		4.....	34	42	46	50	52	56	5.....	38	43	48	49	50	55
		8.....	37	40	48	52	54	62	10.....	37	41	51	46	49	59
		12.....	35	40	52	50	53	65	15.....	37	41	56			
									20.....	37	39	59			
									25.....	36	38	63			
									30.....	33	36	66			
									40.....	33	35	75			
B	{	0.....	34	37	37				0.....	34	37	37			
		4.....	37	39	43				4.....	34	36	40			
		7.....	36	38	45				7.....	34	35	42			
		10.....	37	38	48				10.....	34	35	45			
FULHIO WINTER WHEAT															
	0.....	76	77	77	*	.....	.....								
	5.....	56	58	63	76	78	83								
	10.....	66	67	77	75	75	85								
	15.....	63	64	79	74	74	89								
	20.....	55	60	80	*	.....	.....								

\* Did not head in 90 days.

The experiment using Fulhio winter wheat ran for 90 days following planting. Only vernalized plants headed and flowered. Heading and flowering data are given in table 3. The necessity for a dark-phase following the thermo-phase seems evident. Exposure to a 5-day dark period following emergence and then transfer to a 24-hour photoperiod caused an acceleration of 14 days in flowering, compared with those vernalized plants grown continuously in a 24-hour photoperiod. Ten

dark days required a supplement of sixty-seven 24-hour photoperiods to cause flowering in a total of 77 days, the same as that required by the controls exposed to 24-hour photoperiods continuously. Exposure to darkness of longer than 10 days increased the total time to flower.

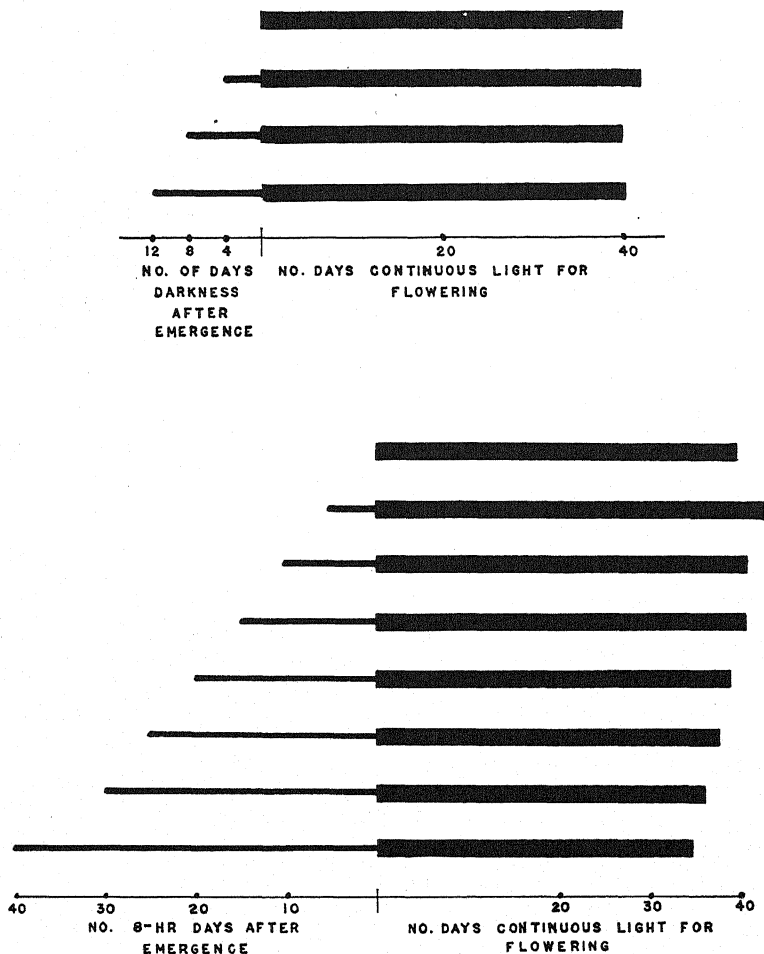


FIG. 1.—Effect of darkness and of exposure to 8-hour photoperiods following emergence on number of days of continuous illumination required to cause flowering in Marquis spring wheat. Narrow lines represent number of 8-hour days or darkness; broad lines, number of days of continuous light. Total number of days from emergence to flowering obtained by addition of the two periods.

#### PHOTO-PHASE OF MARQUIS AND FULHIO WHEATS

The results of the two experiments on the photo-phase of Marquis wheat are given in table 4. The figures for heading and flowering are the average for the first twenty plants to head and flower, except where stated otherwise. Only data

for unvernallized plants are included, as the results of vernalization were not significant.

In experiment A (planted February 24) the adverse effects of low light intensity were shown by retardation or prevention of heading and flowering after transfer to the 8-hour photoperiod. Planting for experiment B was on April 12. The intensity of daylight was much greater, and for that reason the results are probably due to photoperiod. When the plants received fewer than twenty-two 24-hour photoperiods before being transferred to the 8-hour photoperiod, heading and

TABLE 4

HEADING AND FLOWERING DATA OF MARQUIS SPRING WHEAT RECEIVING CONTINUOUS LIGHT FOLLOWED BY 8-HOUR PHOTOPERIODS. PLANTS GROWN CONTINUOUSLY IN 24-HOUR PHOTOPERIOD REQUIRED 35 DAYS TO FLOWER IN EXPERIMENT A; 37 DAYS IN EXPERIMENT B

EXPERIMENT A			EXPERIMENT B			
NO. OF 24-HOUR DAYS AFTER EMERGENCE	ADDITION- AL 8-HOUR DAYS TO HEAD	ADDITION- AL 8-HOUR DAYS TO FLOWER	NO. OF 24-HOUR DAYS AFTER EMERGENCE	ADDITION- AL 8-HOUR DAYS TO HEAD	ADDITION- AL 8-HOUR DAYS TO FLOWER	TOTAL DAYS TO FLOWER
0.....			0.....			
5.....			4.....			
10.....			7.....			
15.....			10.....			
20.....	59*		13.....			
25.....	43	†	16.....			
30.....	41	‡	19.....	§		
			22.....	16	18	40
			25.....	12	13	38
			28.....	8	9	37

\* Only twelve headed.

† Seven flowered.

‡ Five flowered.

§ Only one headed within 45 days.

flowering were prevented or did not occur in 45 days, the duration of experiment B. Those plants receiving twenty-eight 24-hour photoperiods following emergence flowered as quickly when transferred to the 8-hour day as those receiving 24-hour photoperiods continuously, the total flowering time being 37 days in both cases. Apparently, therefore, the continuous light requirement of Marquis spring wheat is completed in approximately 28 days; heading, flowering, and fruiting occurring in an 8-hour photoperiod thereafter as quickly as in a 24-hour photoperiod.

An experiment using 1400 plants failed to detect any substantial portion of the photo-phase of Fulhio winter wheat which can be passed as quickly in an 8-hour photoperiod as in continuous light.

### Discussion

The fact that some seeds, such as *Lupinus polyphyllus*, *Vicia picta*, cabbage, tomato, turnip, swede, and beetroot (13), do not respond to vernalization and

yet the young plants produced do respond to treatment suggests that the thermo-phase is by no means the first phase in the development of the plant from the division of the zygote to the production of seed. During seed ripening it is possible that the phase or phases prior to the thermo-phase may not be completed, and therefore the thermo-phase cannot begin until the prethermo-phase is completed in the young plant. Vernalization treatment would be ineffective until the prethermo-phase was completed.

The length of the thermo-phase has been found to vary, not only with species but also with varieties. DOLGUSIN (2) has subdivided wheats into five groups in respect to the optimum temperature and the time required at those temperatures to vernalize (or complete) the thermo-phase:

GROUP	TEMPERATURE (° C.)	TIME IN DAYS
1. Early spring . . . . .	8-15	5- 8
2. Late spring . . . . .	3-6	10-15
3. Intermediate . . . . .	2-5	20-25
4. Winter . . . . .	1-4	30-35
5. Extreme winter . . . . .	0-3	30-45

Apparently the higher the temperature of vernalization, the shorter its period.

Investigations of KOSTJUCENKO and ZARUBAILO (6), GREGORY and PURVIS (4), and others showed that under special conditions, for instance those prevailing in a cool northern summer and autumn, even the wheats of groups 4 and 5 can be completely vernalized during seed ripening. WORT (17) pointed out that those samples of a variety of spring wheat grown in regions where the ripening temperatures were relatively high responded to treatment to a greater degree than those ripened in a cooler climate, the suggestion being that the thermo-phase had been partially completed in the latter case. That partial completion of the thermo-phase is possible during ripening of the seed has been shown in the experiments described here, but under the conditions of these experiments total completion of this phase during ripening was not accomplished.

MACKOV (12) and EREMENKO (3) report that prolongation of natural daylight with supplementary illumination at night failed to accelerate flowering of wheat if the treatment were supplied during the first week or two after emergence. Subsequent to this initial period both plants exhibited acceleration of flowering by treatment with supplementary illumination. It was concluded that a stage (scoto-phase) exists at which both spring and winter wheats require high temperatures (in contrast with the thermo-phase) and darkness (in contrast with the photo-phase). The work of McKINNEY and SANDO (11) could be interpreted to have established the succession thermo-phase, scoto-phase, photo-phase for winter wheats.

The experiments with Marquis wheat described by the writer failed to detect the presence of the scoto-phase or a phase satisfied by short days immediately



following the thermo-phase. In the case of vernalized Fulhio winter wheat, a definite acceleration of flowering was obtained when the young plants were grown in darkness at  $21.1^{\circ}\text{C}$ . and above for 5 days following emergence from the soil. Here the sequence is thermo-phase, scoto-phase, photo-phase. The possibility of the full or partial completion of the scoto-phase during seed ripening and later during germination must not be overlooked.

Following the scoto-phase, vernalized plants of winter wheat require light and a high temperature. Early work by MELJNIK (LYSENKO, 9) in experiments with

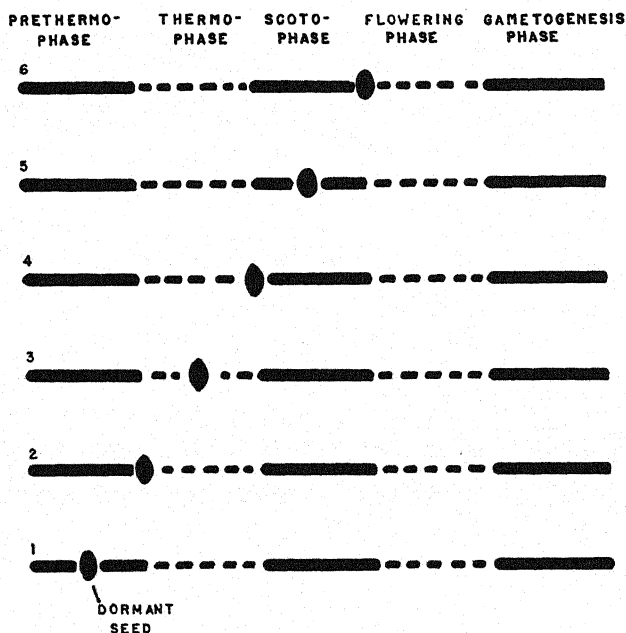


FIG. 2.—Phases of development and possible relation of dormant seed to stages in life history. Various amounts of prethermo-phase, thermophase, and scoto-phase have been completed prior to seed dormancy, as indicated by position of ellipse representing dormant seed. Vernalization of seed would be most effective in 2 and less so in 3, seed of all other classes would not respond.

winter wheat grown at high temperatures showed that vernalized plants grown first in a continuous day for not less than 20 days eared as rapidly in a 10-hour day as those kept in a continuous day throughout; that is, after 20 continuous days long or continuous light is not obligatory, and the long day is indispensable only for a definite part of the period following the thermo-phase (photo-phase). The existence of at least two parts to the photo-phase (gametogenesis phase and flowering phase) was suggested also by KIRICENKO (5) and KRAEVOI (7).

The experiments indicate that such a division of the photo-phase is possible in the case of Marquis spring wheat. The failure of the experiment using Fulhio

winter wheat to detect any portion of the photo-phase which can be passed as quickly in an 8-hour as in a 24-hour photoperiod may have been caused—at least in part—by the low light intensities prevailing during the initial stages of the experiment. Further discussion of the photoperiodic aspects of phasic development may be found in a number of recent papers (1, 8, 10, 14, 15).

Figure 2 graphically summarizes the various phases of development and the possible relation of the stages in the life history of the plant to the dormant seed. No attempt has been made to portray the relative lengths of the various phases.

The complete developmental history, based upon the present literature, may consist of prethermo-phase, thermo-phase, scoto-phase, and a photo-phase consisting of the flowering phase and the gametogenesis phase. A number of these phases may be completed by the time the seed has reached dormancy, depending on the species and variety of the plant and on the environment which acts on the ripening seed. In 1, only part of the prethermo-phase has been completed prior to seed dormancy. Vernalization of such seed would be ineffective, but vernalization of the plants after the prethermo-phase has been passed would be effective. The plants quoted from VASILJEV's paper may belong in this class. In 2 the prethermo-phase has been completed by the ripening seed and the dormant seed is ready to begin the thermo-phase upon germination. Vernalization of such seed would produce the greatest effect.

Not only has the prethermo-phase been completed prior to seed dormancy in 3, 4, 5, and 6, but also varying amounts of the thermo- and scoto-phase. Those seeds which had been partially vernalized during ripening, and which responded to vernalization prior to planting by a smaller acceleration of flowering than those from plants which had not experienced chilling temperatures, doubtless belong to class 3. No response to vernalization of the seed would be expected in 4, 5, and 6, as the thermo-phase has been completed prior to dormancy.

### Summary

1. By chilling the parent plants, the ripening seed of Marquis spring wheat can be at least partially vernalized.

2. No dark-phase or phase satisfied with 8-hour photoperiods immediately following the thermo-phase of Marquis wheat was detected, but exposure of Fulvio winter wheat to a 5-day dark period following emergence and then transfer to continuous light caused an acceleration of flowering compared with those vernalized plants grown in a 24-hour photoperiod.

3. Plants of Marquis spring wheat receiving twenty-eight 24-hour photoperiods following emergence flowered as quickly when transferred to an 8-hour photoperiod as did those receiving continuous light. This suggests that the photo-phase of Marquis wheat may consist of at least two parts, the first requiring long or con-

tinuous day, the second being satisfied with a short or 8-hour photoperiod. No such division of the photo-phase of Fulhio winter wheat was detected, flowering occurring most rapidly in continuous day throughout.

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# RESPONSES OF SUNFLOWER STEMS TO GROWTH-PROMOTING SUBSTANCES

JOHN L. BLUM

(WITH TEN FIGURES)

## Introduction

Histological responses to different growth-promoting substances have been described recently for stems of a number of dicotyledons (2-11) and of three species of *Lilium* (1). It has been established that one such substance, indoleacetic acid, causes the various tissues to react in characteristically different ways in various species of plants. Since the previous investigations include work on responses in stems of several stelar types, but not many observations concerning the differences between the effects of the various substances,<sup>1</sup> it was thought desirable to compare the tissue responses of stems of a single species with what might be regarded as generalized and typical stem structure to a variety of different growth substances. The present study compares the effects of five growth-promoting substances, indole(3)acetic acid, indole(3)butyric acid, indole(3)propionic acid, naphthaleneacetic acid, and phenylacetic acid, on the stems of the sunflower, *Helianthus annuus* L. These acids were selected because they were readily available in crystalline form, and because they had been previously shown responsive in the *Avena* curvature test.

## Material and methods

Sunflower plants grown under greenhouse conditions at various seasons of the year were used for the experiment. The work was carried on throughout 1938 and from January to May of 1939; during this period several different plantings were made, and the plants treated.

The plants were decapitated about 1 cm. above the third node, when the average length of the elongating third internode was somewhat more than 1 cm. for a group of contemporary plants. Treatment in the third internode usually was made 6-7 weeks after planting. A single decapitated plant was treated in one of three ways: (1) by smearing immediately the cut surface with lanolin containing 0.2 per cent growth-promoting substance, a comparatively weak concentration; (2) by smearing with pure lanolin; or (3) by no treatment after decapitation.

Material for histological work was fixed in formalin-acetic-alcohol fixative soon

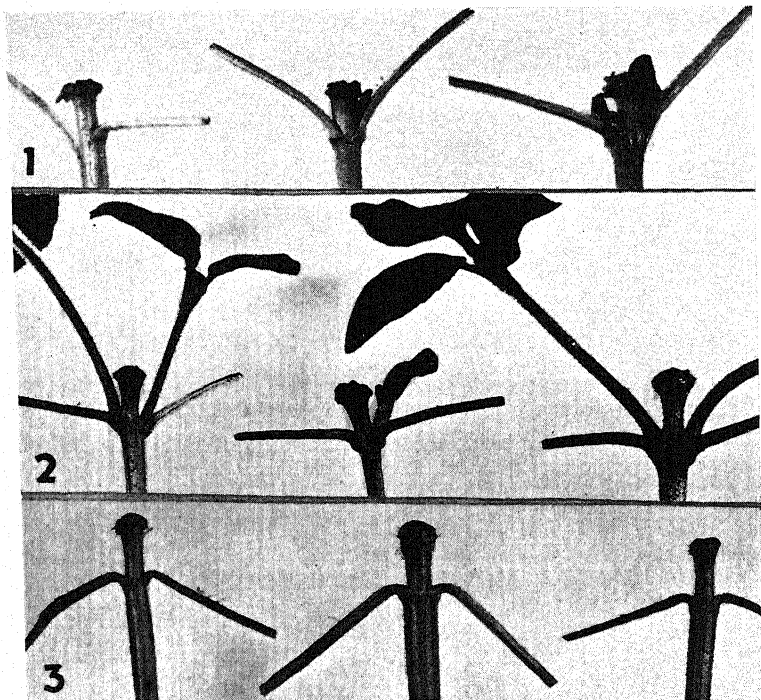
<sup>1</sup> A pertinent paper has appeared since this article was accepted for publication: MULLISON, W. R., Histological responses of bean plants to tetrahydrofurfuryl butyrate. BOT. GAZ. 102:373-381. 1940.

after treatment, and at various intervals up to 35 days. It was imbedded, sectioned, and stained by standard methods.

### Observations

#### GROSS RESPONSES

Within 24 hours after treatment, epinasty of the leaves at the third node is more or less evident for plants treated with indoleacetic (fig. 1) or with indolepro-



FIGS. 1-3.—Fig. 1, sunflower stems 14 days after treatment with indoleacetic acid above third node. Note irregular surface of callus and small axillary branches, development of which has been delayed owing to inhibiting effect of this acid. Blades of leaves removed for convenience in photographing. Fig. 2, 14 days after treatment with indolepropionic acid. Note epinasty of leaves, enlarged stump, and axillary branches. Development of latter inhibited only slightly. Fig. 3, 14 days after treatment with naphthaleneacetic acid. Note epinasty of leaves and lack of axillary branches, development of which has been completely inhibited.

pionic acid (fig. 2), and very conspicuous with naphthaleneacetic (fig. 3) and indolebutyric acid. Phenylacetic acid mixture induces little if any epinasty, even after several days. Epinastic bending of any one petiole does not increase markedly after a period of 1 day following treatment, and epinastic bending of leaves at the second node was never observed.

Treatment in the third internode does not occasion responses below the third node. Most of the responses (except for externally visible ones at the node) are confined to the callus and the upper 3-4 mm. of the decapitated stump of the stem from which, following application of the growth substance, the callus is formed. The region within 2-3 mm. above the node remains essentially unchanged from the time of decapitation and treatment. This permanent localization of the response in sunflower is of interest in light of work on the bean, which has been shown to respond, within 128 hours after treatment, as far as 5 cm. below the point of application (7).

Within 5-10 or more days after treatment the decapitated ends of the stems assume characteristic shapes, which are specific to a certain extent for each of the acids. Following application of indoleacetic, indolebutyric, naphthaleneacetic, and phenylacetic acid, growth is largely in the callus which develops above the former position of the cut surface, and the vertical sides of the decapitated stump remain approximately unchanged in outline. With indolepropionic acid there is an externally observable response below the cut surface, in that the upper portion of the stump swells considerably. In the uppermost 5-6 mm. of the stump, the amount of swelling as a result of application of this acid is about inversely proportional to the distance below the surface of decapitation, resulting in a stump which tapers from apex to base. The upper surface of a callus formed after the application of indoleacetic acid is frequently quite irregular; with the use of the other acids it is more commonly smooth.

Adventitious root tips appeared about 12 days after treatment at the outer edge of the apex of many of the tumors formed following treatment with indoleacetic acid. These roots never grew more than a few millimeters under greenhouse conditions. Although the sunflower forms such roots after indoleacetic acid has been applied, they are formed only occasionally after application with indolebutyric and indolepropionic acid, and never with naphthaleneacetic or phenylacetic acid. New roots were not formed to any extent after a period of 3 weeks following treatment with any of the acids.

Plants decapitated in the third internode and smeared with pure lanolin, or left untreated, form axillary branches at the third node, and sometimes the second node also, within 2 weeks. Certain of the acids inhibited development of the axillary buds from which such branches come. Counts have not been made on a large scale, but results from limited plantings indicate certain differences in the acids, in the extent to which they inhibit the development of axillary branches. Indoleacetic (fig. 1) and indolebutyric acid seem to inhibit bud development for a time, and any branches which develop appear relatively later or in fewer numbers than those produced by plants untreated or treated either with pure lanolin, indolepropionic acid (fig. 2), or phenylacetic acid. The latter substances appear to inhibit

bud development only slightly, if at all. Treatment with naphthaleneacetic acid causes complete inhibition of bud development (fig. 3). With one exception, plants treated with this acid did not produce axillary branches at any of the three nodes. This is in accord with the findings of MITCHELL and STEWART (10), who applied the same acid to decapitated stems of bean plants.

#### ANATOMY OF UNTREATED STEM

The sunflower stem as it appears normally (figs. 4, 5) is characterized by a cylinder of fifteen to thirty primary vascular bundles. Secondary thickening due to cambial activity is beginning or has begun at the time of treatment, and if the stem is allowed to grow normally, will develop a continuous vascular cylinder, interrupted at intervals by uniseriate rays. The large hairs borne on the epidermis may be one channel for conduction to the cortical cells of materials applied in lanolin, since it is difficult to smear the decapitated end of a stem and keep the numerous and sizeable hairs which subtend the cut surface entirely free of the mixture. The untreated stem is so generally familiar that detailed description is unnecessary.

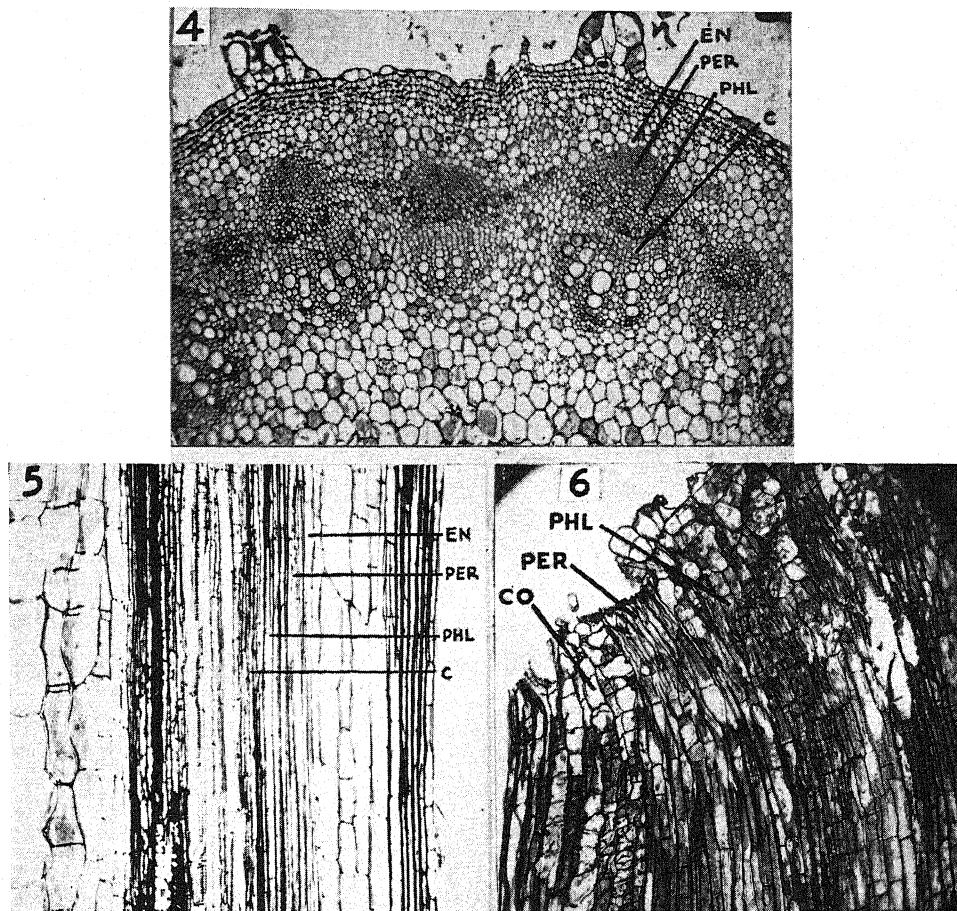
#### ANATOMY OF TREATED STEMS

Sunflower stems which were decapitated and left untreated, or were smeared with pure lanolin, did not change materially in histological structure in the 3-4 weeks following treatment during which observations were made, except for shrinkage and loss of color and drying out of the exposed portions of those untreated. In such controls, secondary thickening and cell maturations were apparently suspended, and no appreciable callus tissue was formed. Other workers have shown that similar control stems of tomato (2), cabbage (3), and species of *Iresine* (6), *Lilium* (1), and *Mirabilis* (4), as in the sunflower, will not proliferate; but that those of bean will proliferate extensively and form considerable callus (7).

Certain of the parenchymatous tissues near the smeared surface, shortly after treatment with one of the substances, begin to proliferate. Cells of the tissues which are not responsive (for example, the epidermis, the pericycle, and the tracheids and vessels of the xylem) are not ordinarily broken and carried upward by this development but remain in their original position (fig. 6) and undergo some crushing. A single primary xylem vessel will thus appear to end blindly at its apical extremity in a mass of cells which have formed from proliferation in an apical direction of the xylem parenchyma around the vessel.

The pith is responsive to all the acids for some distance below the cut surface. The cells immediately below the cut surface at the time of decapitation divide and elongate rapidly, so that most of the callus tissue develops from the uppermost layers of cells. Divisions of cells within a few cell layers of the cut surface may not be followed by cell enlargement, with the result that a single pith cell will divide

and its daughter cells redivide many times, and all the daughter cells will occupy a region conforming in position and outline with that of the original parent cell. Other pith cells, or cells from the division of pith cells, mature into wound tra-



FIGS. 4-6.—Fig. 4, cross section in third internode of normal stem at time of treatment: *c*, cambium; *en*, endodermis; *per*, pericycle; *phl*, phloem. Fig. 5, longisection in third internode of normal stem at time of treatment. Fig. 6, longisection near apex of stem 4 days after treatment with indolebutyric acid: *co*, cortex; *per*, pericycle; *phl*, phloem. Note inactivity of pericycle and proliferation within phloem and cortex.

cheids. Such tracheids appear to bear no functional relation with one another, or they may be organized into short, bundle-like, vertically oriented groups, which end blindly at both ends in the parenchyma of the callus. Occasionally such tracheids connect with cells of the secondary xylem in the region of the ray.

Cells of the perimedullary zone or of the primary xylem parenchyma, in con-



trast to the pith, do not mature into such xylem cells, but by division and vertical elongation contribute to the formation of the callus.

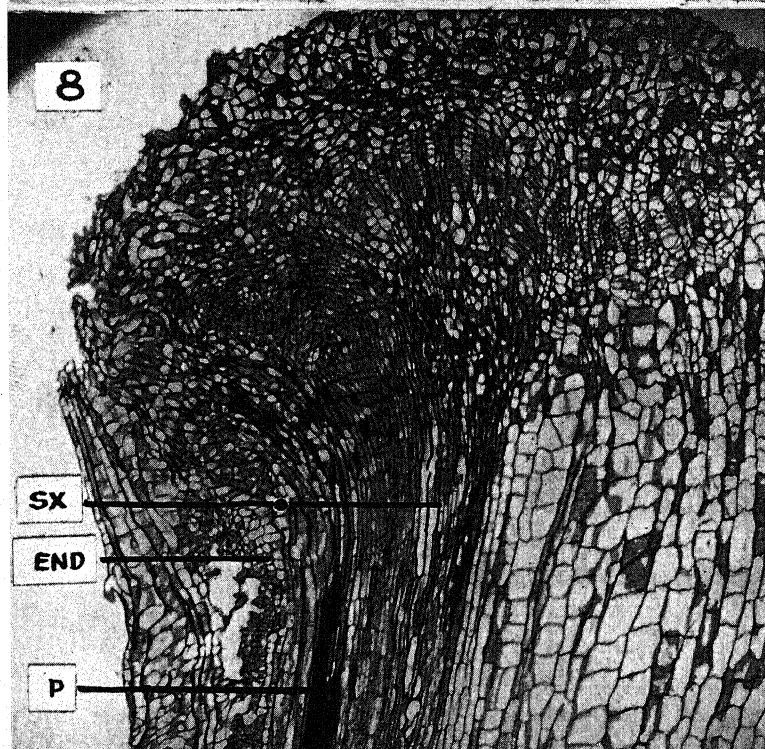
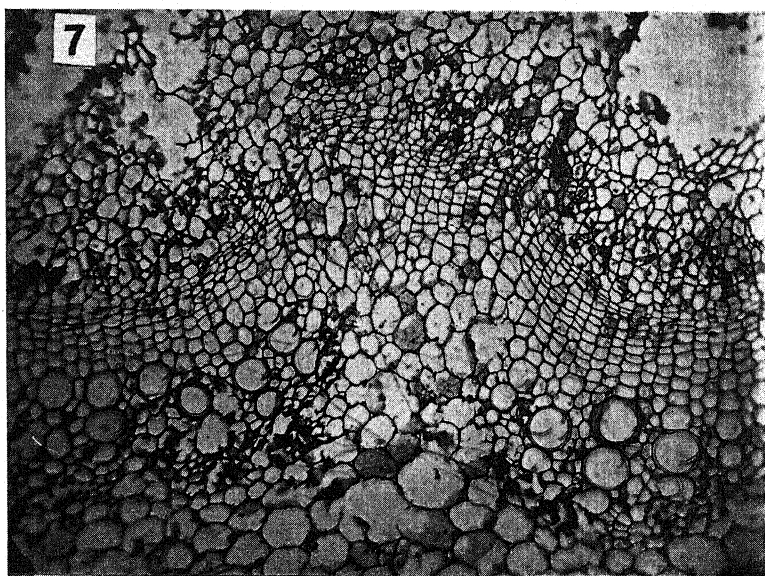
The cambium is highly reactive to naphthaleneacetic acid and to a less degree to all the other acids. In the proliferation of its cells nearest the cut surface, the cambium is believed to contribute to the developing callus, and its normal activity in secondary thickening is increased in the uppermost 3-4 mm. of the stump. With naphthaleneacetic acid there is a prominent increase in the formation of secondary xylem, the stump of the third internode becoming quite woody within 3-4 weeks after treatment. A similar response has been shown to follow treatment of other species of plants with naphthalene acetamide (8, 10). The degree of response in sunflower decreases from the cut surface down, so that the amount of secondary thickening 1-2 mm. above the node, if any, is about equal to that below the node, or to that of the third internode of an uncut and untreated control plant of comparable age.

Secondary thickening in normal plants, and in plants treated with any of the acids except phenylacetic, is more or less uniform for all regions upon the circumference of the cambium, and the cambium itself will appear in cross section as an approximate circle. With phenylacetic acid, however, there is greater cambial activity between the bundles than within them, resulting in a cambium cylinder which is vertically ridged in the region of the ray, and which appears in cross section for a few millimeters below the cut surface to be distended outward between two adjacent vascular bundles (fig. 7). This interfascicular cambial activity is not so great as that of the cambium after treatment with naphthaleneacetic acid, and it never causes the stem to become especially woody.

The parenchyma of the primary and secondary phloem is moderately reactive to all the acids (fig. 6). In stems treated with indoleacetic acid it contributes to the formation of the callus by cell division and elongation, and indirectly (through its derivatives in the callus) to the organization of adventitious root tips. The latter project externally from the callus near the cut surface. The development of such roots in other species of plants has been described in detail by previous workers, who have likewise traced the origin of the roots to the phloem parenchyma (2, 3, 5). All such roots do not develop entirely from the phloem in sunflower, and it has been indicated that comparable roots in *Pisum* may develop from a meristematic region which itself arises from derivatives of many cortical and stelar tissues (11).

The pericycle over the bundles, already thick walled at the time of treatment, has not been found appreciably responsive with any of the acids, either in cell division or in cell enlargement (fig. 6).

The endodermis is highly responsive for 1-2 mm. below the cut surface to at least naphthaleneacetic, indoleacetic, and indolebutyric acid, its cells reproducing by radial divisions and elongation rows of isodiametric or radially elongated cells



FIGS. 7, 8.—Fig. 7, cross section 0.5 mm. below upper exposed surface of callus in third internode of stem 18 days after treatment with phenylacetic acid. Note cambium distended outward between the two bundles. Fig. 8, longisection near apex of stump of stem 12 days after treatment with naphthaleneacetic acid: *end*, cells proliferated from endodermis; *p*, pericycle; *sx*, secondary xylem. Note lacunae in cortex.

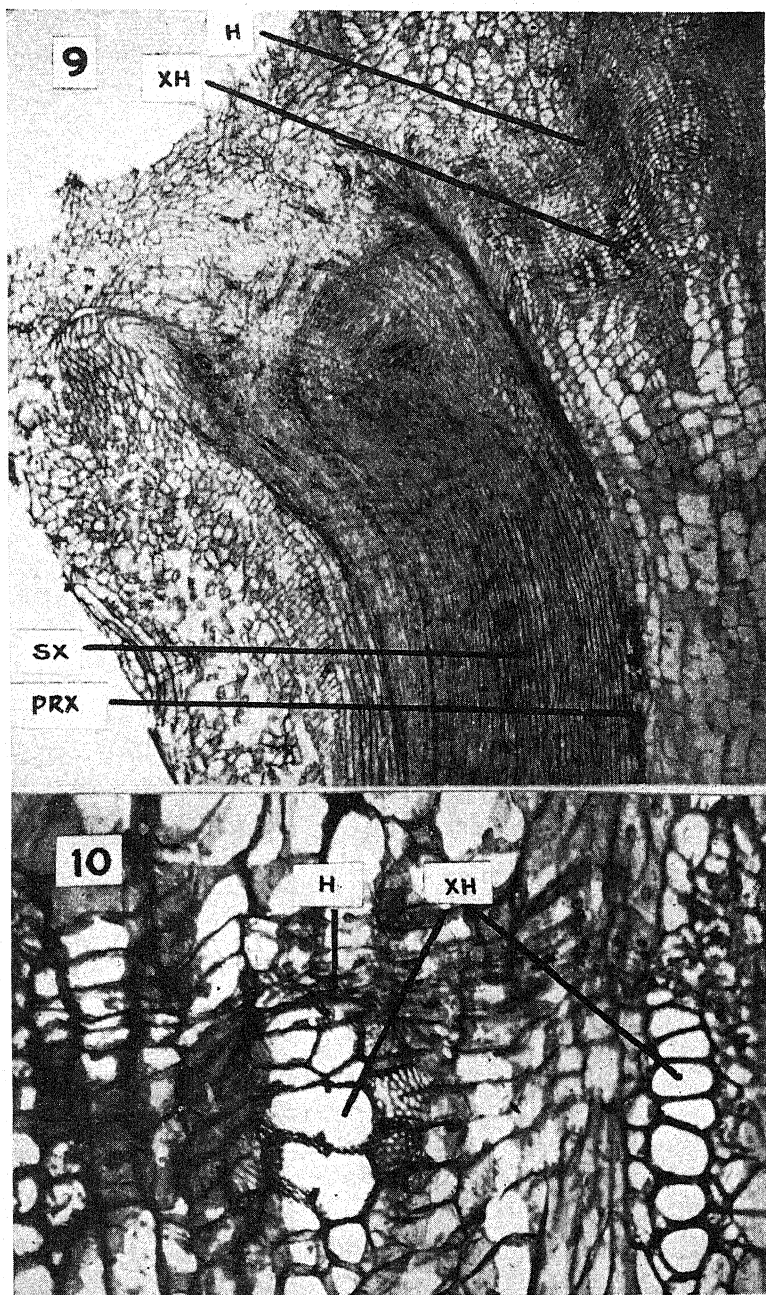
(fig. 8). Owing perhaps to unequal growth, individual rows become detached from those of rows of similar origin which are adjacent, and intercellular spaces are formed. These cells push against the outer cortical cells, forcing the latter (and hence the epidermis) outward, and in part account for any irregularity in the sides of the stump. Large lacunae develop in the cortex (fig. 8) following this rupture. Such lacunae may be due to disintegration of the middle lamella of certain of the cells, as suggested by SCOTT (11) for similar lacunae in auxin-treated stems of *Pisum sativum*. In the development of this endodermal mass of cells in sunflower, the casparian strips, when they can be traced, remain with the innermost cell of each radial row, so that the endodermis can be said to retain its identity. With indolepropionic acid there is considerable cell division in the cortex, but no lacunae have been observed.

The cortex external to the endodermis responds to any of the acids used by cell divisions and enlargements near the surface of application, and since the epidermis shows no response whatever, forms what is seen in cross section as the entire periphery of the callus. A characteristic cortical response is shown following treatment with indolepropionic acid, the cortical cells (and in part the pith) enlarging radially and evenly in proportion to their proximity to the surface of application to form the characteristic tapering stump just described, and illustrated in figure 2.

The formation of a corklike layer from the outermost cortical cells has been observed occasionally with the use of naphthaleneacetic acid. Sometimes this appears several millimeters below the cut surface.

In considering the responses shown in the cortex, it may be observed that, with indoleacetic acid, many of the adventitious root tips appear to be derived from meristematic cortical tissue alone. Development of these roots was not followed in detail, since they were rather infrequent in occurrence.

One activity evident within the stems in response to the application of growth-promoting substances has been set apart for discussion separately. This activity is the development of a horizontal cambium-like tissue (figs. 8-10) within the callus and in the plane of (or above) the cut surface, from cells which have proliferated from the xylem parenchyma and the pith. The horizontal "cambium" is continuous with the true cambium where the latter was cut across in decapitation, and it forms a platelike meristem over the surface of decapitation. It produces cells on its lower face which mature into xylem (figs. 9, 10) and on its upper face cells which mature into a tissue which is sometimes phloem-like in appearance. The immature xylem cells produced by this "cambial" activity elongate in a horizontal direction and become lignified with reticulate thickenings. The long axis of the cell after elongation may apparently be in any direction from the radial to the tangential. Thus there is formed across the stem, below the horizontal meristem, an unorganized mass of xylem cells in groups, all of which may be oriented in different directions. The horizontal cambium is not an entirely flat plate. It is more



FIGS. 9, 10.—Fig. 9, longisection at apex of stem 25 days after treatment with naphthaleneacetic acid: *h*, horizontal “cambium”; *prx*, primary xylem; *sx*, secondary xylem; *xh*, group of tracheids formed toward lower face of horizontal cambium. Note extreme development of secondary xylem, and lacunae in cortex. Fig. 10, longisection at cut surface of stem 14 days after treatment with indoleacetic acid: *h*, horizontal cambium; *xh*, groups of cells matured into tracheids after having been cut off toward lower face of horizontal cambium.

appropriately a plate whose circumference forms an approximately plane circle, but whose central portion is irregularly bent, warped, or dented (fig. 9). Its initials are developed successively, soon after treatment, in the cells formed from upward proliferation of the true cambium, the xylem parenchyma, and the pith. The initials can be traced internally over the cut ends of the xylem vessels several layers from the upper free surface of the callus. In early development the horizontal cambium lacks a central portion. The edges, approaching the center of the stem from all points on the circumference of the true cambium cylinder, may eventually close over the central region, perhaps uniting with previously isolated meristematic regions in the pith. The closing over in the center of the callus may or may not occur; when it does occur, it appears usually to be completed by about the twentieth day after treatment. Development of the horizontal cambium has been observed most conspicuously and most constantly with naphthaleneacetic acid; it has been observed less regularly following application with the other acids.

### Discussion

These results point toward some degree of specificity for each of the various growth-promoting substances used in regard to the responses which result from their application. Following the application of indoleacetic acid, development of adventitious roots from the tumor was frequently observed. Such roots regularly were not formed in response to treatment with any of the other acids. Partial inhibition of the development of axillary branches at the third node occurs in response to treatment with this substance and to treatment with indolebutyric acid. Certain other responses following the application of indoleacetic are not specific for this acid, that is, similar responses occur after treatment with many or all of the other acids used.

Plants treated with indolebutyric acid have not been found to show distinctive responses and are distinguished from plants treated with the other acids on the basis of negative characters. The latter plants in all cases show responses which do not occur following the application of indolebutyric acid.

The typical response in stems which have been treated with indolepropionic acid is the growth in diameter of the apical end of the stem. This growth is due mostly to a characteristic enlargement of cells of the cortex and pith, the enlargement being preceded by divisions of cells only 1-2 mm. below the cut surface. Application of indolepropionic acid will inhibit the development of axillary buds only slightly, and in this respect also its effects can be distinguished from those of the other substances used.

Following treatment with naphthaleneacetic acid marked responses occur. Epinasty of the leaves at the third node is usually strongly evident within 24 hours after application. Cambial activity is stimulated by this substance more than by any of the other four used, and the stump becomes relatively woody in a short

time. The development and activity of the horizontal "cambium" is also markedly stimulated. One of the most sharply distinguishing responses to this treatment is the complete inhibition of bud development in the axils of the leaves at the third node.

The only response characteristic and specific for plants treated with phenylacetic acid is the stimulation of the activity of the interfascicular cambium, this activity resulting in a cambium cylinder which is distended outward between the vascular bundles.

It is to be emphasized that specific responses of an individual stem to treatment with any of these acids can never be predicted with certainty. The responses have been found usually to occur as described, so that a group of stems which have responded to treatment with indolepropionic acid, for example, can be identified and distinguished as such from groups of stems treated with any of the other four acids. But it is possible to select from the group atypical individuals which, taken alone, might appear both externally and histologically more like an indoleacetic tumor, or a phenylacetic tumor, or a composite of these, than like other members of its own group. There has been also considerable variation in the time elapsing between treatment and response, and in the degree of final response. It would appear, therefore, that a study based upon averages over a very large population of treated plants should be made of such responses. The present study has been concerned with the late or final effect of the stimulants applied, rather than with the development of these effects in detail over the period of time between treatment and completion of the experiment.

### Summary

1. Sunflower plants were treated upon a decapitated surface in the third internode with one of five growth-promoting substances in lanolin.
2. Certain responses are common to treatment with all of the acids. Among these responses are: the formation of parenchymatous or meristematic callus tissue at the exposed surface of the pith, of the parenchyma of the xylem and phloem, and of the cortex, especially the endodermis; and increased secondary thickening near the apex of the stump. The conducting elements of the xylem and phloem, the pericycle, and the epidermis do not react to any of the acids.
3. A horizontal cambium-like tissue is formed soon after treatment, which connects with the true cambium at the cut edges of the latter and forms a platelike meristem across and within the callus. Cells which it forms to the lower side develop into tracheids.
4. Some of the acids bring about characteristic and specific responses, as follows:
  - a. Indoleacetic acid causes the partial inhibition of bud development at the third node and the formation of adventitious roots.

b. Indolebutyric acid causes partial inhibition of bud development, but it does not cause general formation of root tips, extreme secondary thickening, marked cell enlargement, nor marked activity of the interfascicular cambium.

c. Indolepropionic acid inhibits bud development slightly if at all, and its application results in a characteristic stump which tapers from apex to base, owing to a gradient of cell enlargement, chiefly in the cortex and pith, and near the uppermost portion of the tumor to cell divisions.

d. Naphthaleneacetic acid causes marked epinasty, completely inhibits bud development, causes extreme development and differentiation of secondary xylem, and marked development and activity of the horizontal "cambium."

e. Phenylacetic acid induces in the third internode disproportionate activity of the interfascicular cambium, the cambium becoming outwardly distended between the vascular bundles.

This research was carried on at the University of Wisconsin. The writer acknowledges gratefully the time and assistance given by Professor EMMA L. FISK in the course of the work.

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# FLORAL ANATOMY AND MORPHOLOGY OF ANEMOPSIS CALIFORNICA

CONTRIBUTIONS FROM THE HULL BOTANICAL LABORATORY 524

CHARLES H. QUIBELL

(WITH THIRTY-EIGHT FIGURES)

## Introduction

This paper describes the vascular supply to and in the individual flower in the inflorescence and also the development of the ovule and megagametophyte. *Anemopsis californica* (Nutt.) H. and A. is a moisture demanding, alkali tolerant, perennial herb of the southwestern United States and northern Mexico, for which a new variety *subglabra* has been recently established by KELSO (7). The material used here is of that variety.

*Anemopsis* is a member of the Saururaceae, frequently included as a tribe in the Piperaceae. On the basis of anatomical considerations, ROUSSEAU (8) has reviewed the systematic treatment of the Piperales and concludes that *Saururus* is more closely related to *Piper* than the latter is to *Peperomia* of the Piperaceae. HOLM (3) has described the vegetative morphology and anatomy of the species, including the anatomy of the involucre and floral bracts. SCHMITZ (9) has described the development of the flower and vascular anatomy of several species of the Piperales but not of *Anemopsis*. JOHNSON (6), in a paper dealing with the relationships of the order, has reviewed the development of four genera already investigated and added data on four more, including *Anemopsis*. Of this he reports that "the functional megaspore is one out of two potential megaspores. . . . A tapetum is formed and is persistent. . . . The mature embryo-sac is a typical seven-nucleate one. . . . The first division of the endosperm nucleus is followed by a cell wall cutting the embryo-sac into an upper and a lower cell . . . it is the upper one of these two primary cells that divides further to form a considerable mass of endosperm. The lower cell forms an elongated flask-shaped haustorium with but a single nucleus." JOHNSON does not elaborate these statements and his paper is not illustrated. HÄUSER (2) interprets JOHNSON's statement with regard to two megaspores to mean that after the first division of the megaspore mother cell only the lower nucleus divides again and the megagametophyte is derived from the two megaspores resulting from such division. On the basis of the same report, SCHNARF (11) assumes tentatively that the megagametophytic development is of the *Scilla* type. The observations reported here confirm SCHNARF's assumption. A 16-nucleate megagametophyte derived from four megaspores has



been reported for *Peperomia pellucida* (1, 5). The investigations stimulated by this discovery have been summarized by SCHNARF (10, 11). Although all the seventeen species of *Peperomia* which have been studied possess 16-nucleate megagametophytes, and other 16-nucleate megagametophytes have been discovered in several unrelated families, no other genus in the Piperales exhibits megagametophytes with more than the usual number of eight nuclei. These megagametophytes are derived, in different genera, from one, two, or four megasporos.

### Material and methods

The material was collected at various stations near Fresno, but chiefly in a meadow along Kings River above Centerville, California. The inflorescences, or more frequently sections of them, were killed and fixed in a variety of fluids, of which an alcohol-formalin-acetic acid mixture proved most satisfactory. Heidenhain's haematoxylin-orange G combination was satisfactory.

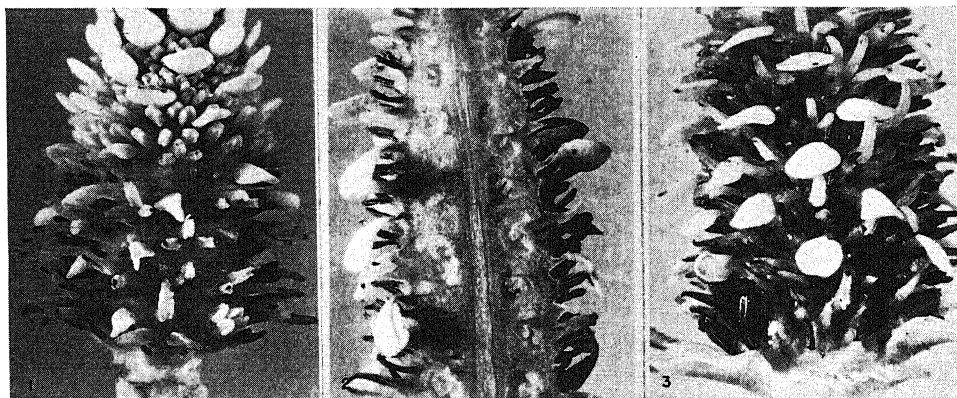
### Observations

**INFLORESCENCE AND FLOWER.**—The thick horizontal rhizome is perennial, a rosette of leaves expanding from its apical bud each spring. From the axils of some of these leaves arise long stolons. Vegetative propagation seems to be the chief means of distribution, few seedlings having been observed. From the axils of other leaves rise single flowering stems whose apices are at first surrounded by the single cauline bract borne on these stems. The cylindrical or slightly conical primary inflorescence, pushed up out of this enveloping bract by the growth of its peduncle, is then protected by the whorl of 6–8 white involucre bracts which are turned up over it. When these spread out horizontally, the inflorescence is still covered for a short time by the small floral bracts, one subtending each of the numerous, spirally arranged flowers, except the basal ones just above the involucre bracts. The horizontal spreading of these bracts is acropetal (fig. 1).

The individual flowers, when thus uncovered, for a brief period show only the closely packed, turgid tips of the six yellow anthers. Subsequent growth spreads the anthers, uncovering three grooved stigmas, whose growth then brings their tips well above the anthers (fig. 1). The ovary has a single locule and three parietal placentae. It is completely inferior, so that the stigmas and the nearly sessile anthers project from the general surface of the inflorescence axis (fig. 2). Each of the three stigmas is grooved the full length of its inner face. These three grooves are united at the surface of the inflorescence axis to form an actual or potential pore leading into the locule. According to JEPSON (4) there are 6–10 ovules on each placenta or 18–30 ovules per flower. Counts of thirty flowers (excluding those near the apex of the inflorescence) distributed among six heads averaged 8.5 ovules per flower. The arrangement of two stamens opposite each carpel is shown in

figures 1 and 3. Two or three leaves arise from a meristem in the axil of the cauline bract and sometimes one or two secondary inflorescences arise in their axils.

**VASCULAR SUPPLY OF INDIVIDUAL FLOWER.**—The vascular bundles of the axis of the inflorescence are arranged in an irregular ring. The vascular supply of the individual flower is a single bundle (fig. 4 X) which, at the level of the next flower directly below, swings outward from the ring of bundles, rises parallel to it until the base of the flower is reached, and then is divided into seven bundles, all of which are turned outward around the locule (figs. 5-8). At a point level with the top of the locule six of these seven bundles undergo a series of divergences and



FIGS. 1-3.—Fig. 1, lower three-fourths of inflorescence. Fig. 2, longisection showing ovaries surrounded by inflorescence axis. Fig. 3, similar to and somewhat older than inflorescence in fig. 1.

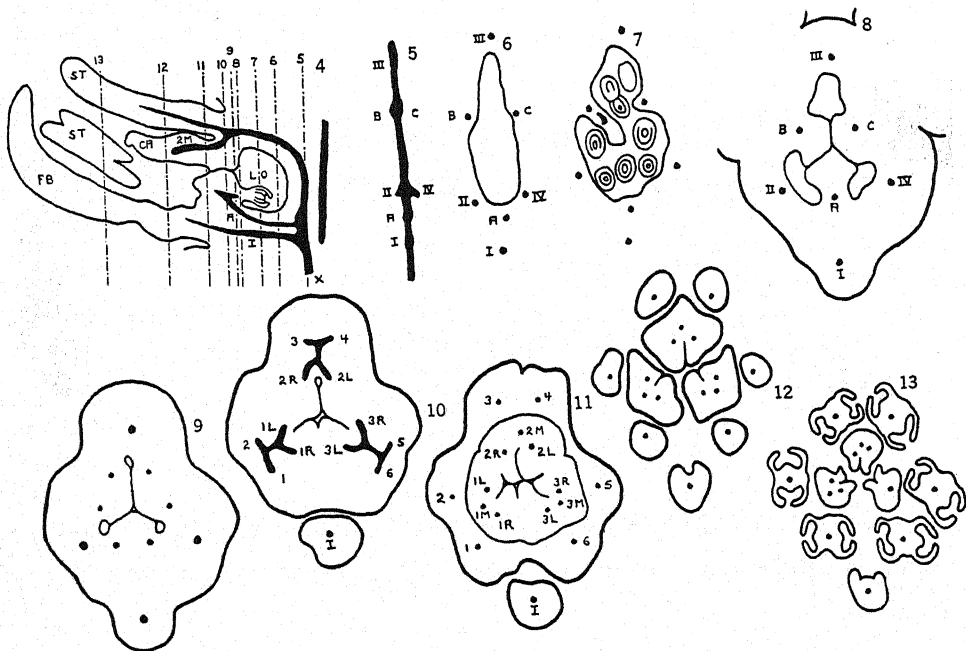
anastomoses (figs. 4, 9-11) which result in fifteen bundles supplying the filaments and styles (figs. 12, 13).

The bundle of the bract (*I*) is the first divergence from the main supply bundle, and without anastomosing with any other it provides the entire vascular supply for that organ. The lower placental bundle (*A*) is the next divergence, followed in turn by two paired divergences, the lower stamen bundles (*II*, *IV*) and the upper placental bundles (*B*, *C*), and by the upper stamen bundle (*III*) which extends out over the upper side of the locule.

The placentae are parietal and extend little more than half way down the locule wall (fig. 2). Above them (level with the top of the locule) the placental bundles split in two, diverge right and left at sharp angles, and then ascend as lateral bundles in the two corresponding styles. The stamen bundles, one opposite each carpel, extend around the locule to a point somewhat above its top, are split trichotomously, and diverge at sharp angles. One branch extends inward and is the central bundle in the style, while the other two spread right and left and extend into the two stamens associated with the carpel. The central bundle of each style,

at the point where it is turned upward into it, is usually connected by horizontal branches with the two styler laterals.

There are occasional variations from this pattern. The basal flowers in the inflorescence lack floral bracts. No instance of variation in the number or arrangement of the six placental and stamen bundles has been observed, but variations in the fifteen divergences from them are comparatively frequent. A relatively large proportion of flowers lack one or more stamens or a style. Short abortive



FIGS. 4-13.—Fig. 4, longisection of flower bud in inflorescence still inclosed by involucre bracts. Figs. 5-13, cross sections of flower in bloom, at levels corresponding to those indicated by numbered lines on fig. 4. *FB*, floral bract; *ST*, stamen; *CA*, upper end of carpel; *LO*, locule and one ovule; *I*, floral bract bundle; *II*, *III*, *IV*, stamen supply bundles; *A*, *B*, *C*, placental bundles; *1*, *2*, *3*, *4*, *5*, *6*, stamen bundles; *1R*, *1M*, *1L*, *2R*, *2M*, etc., right middle, and left styler bundles of carpels *1*, *2*, and *3*.

styles, lacking stigmas, have their middle bundles diverged from the corresponding stamen bundle, but they do not have lateral bundles, which if diverged at all from the corresponding placental bundles form only short vestigial traces which do not extend into the style base. All instances of abortive stamens showed at least some anther development and vascular bundles. Possibly those abortive stamens which consist of a short sharp-tipped filament, with no vestige of anther development, would have no vascular bundle.

A doubling of all the lateral styler bundles was observed in one of the three flowers examined in each of two inflorescences. The extra bundles were short,

derived by splitting of the divergences from the placental bundles, and terminated at about the level of the anastomoses between the main and lateral bundles in or just below the stylar base. They are not involved in this anastomosis. The anastomoses between main and lateral stylar bundles are frequently incomplete, but in no flower observed were they absent in all styles. Occasionally a placental bundle is diverged into three instead of two, and the extra one is connected with the base of a middle stylar bundle.

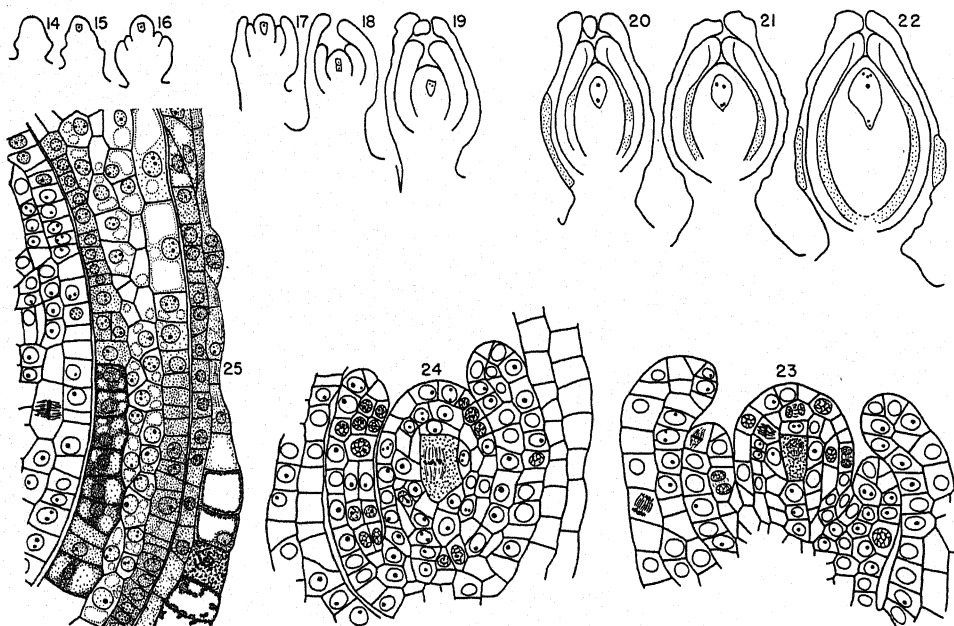
**INTEGUMENTS.**—The outer integument arises first, followed so quickly by the inner that the latter for a brief period may overgrow the former (figs. 14-16). Until the four megaspore nuclei have been formed, the growth rate of the outer integument exceeds that of the nucellus, so that a large space is developed above the latter (figs. 17, 18). As a result of change in relative growth rates during the prolonged 2-nucleate phase of the megagametophyte, this space is occupied by the nucellus and the rim of the inner integument (figs. 19, 20), and subsequently the growth rates of the integuments and nucellus keep pace with each other (figs. 21, 22). Both integuments are two cell layers in thickness in the beginning (fig. 23). In the outer integument the number usually does not increase except at its base and apex, when development of the embryo is well advanced. In the inner integument a third layer results from periclinal divisions in the inner layer, beginning about the time the megaspore mother cell divides (fig. 24). A fourth layer is more or less completely formed owing to doubling of the middle layer, when the megagametophyte is 8-nucleate (fig. 25). The largest cells of the ovule occur in the outer layer of the inner integument opposite to and above the megagametophyte. These cells are three or more times as long as broad, even as early as the end of the 2-nucleate phase (figs. 20, 25). During the latter half of this phase the cells of the inner layer, opposite and below the megagametophyte, grow much larger in both dimensions than those in the upper part. Beginning in isolated cells, the protoplasts of this portion of the layer all take a deep stain by the end of the 4-nucleate phase. In post-fertilization stages even the thinner portion of the layer over the top of the nucellus stains in this manner (figs. 20-22, 25).

In the outer integument the cells of the outer layer become much shrunken and stain weakly, so that their end walls are difficult to distinguish; but there are occasional short vertical rows of much enlarged cells which protrude from the surface of the ovule and later stain deeply like those of the inner layer of the inner integument (figs. 20, 22, 25).

**NUCELLUS.**—The apex of the nucellus, above the point of origin of the inner integument, is at first small and rounded (fig. 15), but by the time the rim of the outer integument passes beyond this apex the median longitudinal section of the nucellus is a truncated oval or ellipse (fig. 17). It shows little subsequent change in size or shape until after the megaspore mother cell divides, although in the

meantime the integuments and the ovule base have grown considerably (figs. 17, 18). During the 2-nucleate phase of the megagametophyte the shape of the nucellus, in longitudinal section, becomes a considerably less truncated ellipse (figs. 18–20). Its growth in length is most pronounced below the megagametophyte, with the result that the latter exchanges its original central position for one in the upper half of the nucellus.

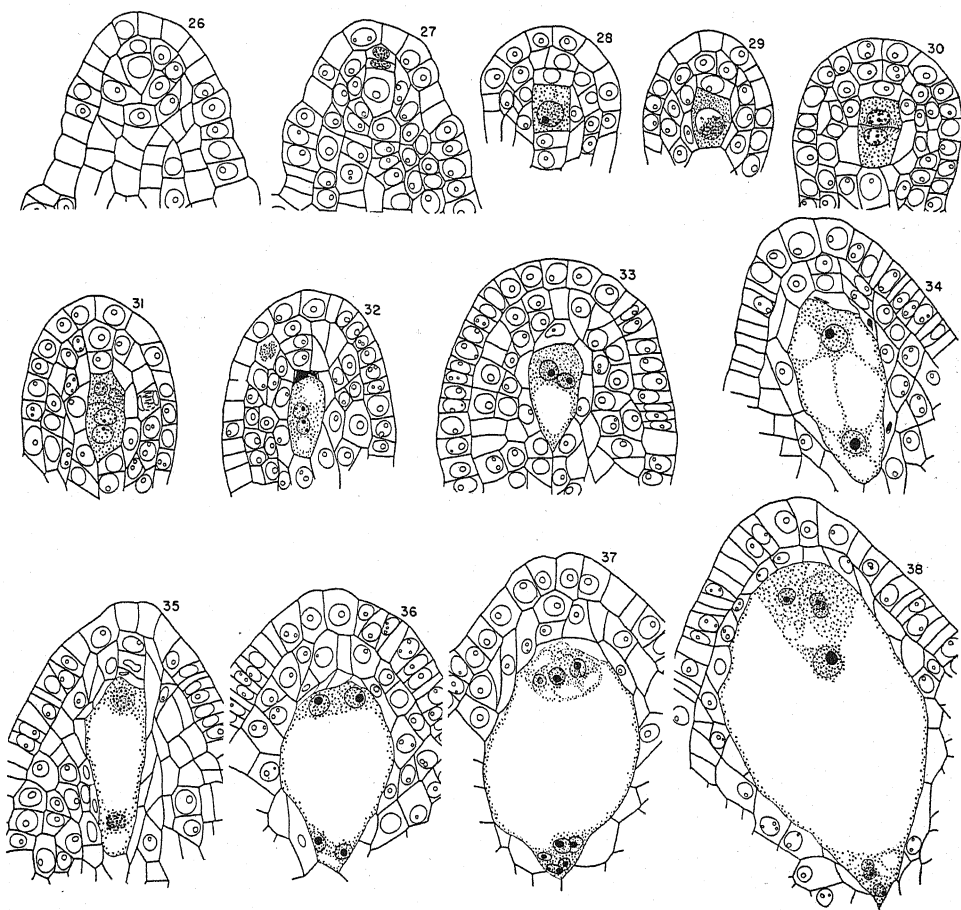
The megaspore mother cell and two parietal cells arise from a hypodermal primary sporogenous cell. These three cells are recognizable in some ovules when



FIGS. 14–25.—Successive stages in ovule development. Megaspore mother cell present in fig. 15 and in prophase of meiosis in figs. 16 and 17; first meiotic division complete in fig. 18; 2-nucleate stages of megagametophyte in figs. 19 and 20, 4- and 7-nucleate stages in figs. 21 and 22. Figs. 23–25, stages in integument development from prophase of meiosis (fig. 23) to 8-nucleate phase of megagametophyte (fig. 25).

the inner integument is initiated (figs. 26, 27). During the prophase of the first meiotic division the outer parietal cell frequently undergoes anticlinal division. A periclinal division of this cell or any division of the inner parietal is rare (figs. 23, 24, 28, 29). Subsequent encroachment of the 2-nucleate megagametophyte upon the parietal cells is steadily balanced by their growth and division tangentially until the megagametophyte nuclei divide, after which frequently only one nucellar cell intervenes between megagametophyte and epidermis (figs. 32–38).

**MEGASPORE MOTHER CELL.**—This cell begins the protracted prophase of the



FIGS. 26-38.—Fig. 26, megaspore mother cell and primary parietal cell; inner integument just starting. Fig. 27, primary parietal cell divided; megaspore mother cell still differentiated only slightly, except in size. Fig. 28, megaspore mother cell in prophase of meiosis. Fig. 29, same in synizesis. Fig. 30, first meiotic division completed. Fig. 31, four megaspores in two cells after considerable growth has occurred in chalazal pair. Fig. 32, late stage in degeneration of micropylar megaspores. Fig. 33, 2-nucleate megagametophyte following resorption of micropylar megaspores. Fig. 34, nuclei at poles and in position for division; megagametophyte and nuclei slightly larger than average. Fig. 35, anaphase of division in 2-nucleate megagametophyte; axis of spindles at right angles to that of megagametophyte and to each other; chalazal division figure complete; one set of chromosomes of micropylar figure present in next section. Fig. 36, 4-nucleate megagametophyte. Fig. 37, polar nuclei about to fuse; egg to left. Fig. 38, 7-nucleate megagametophyte; egg nearer to observer than synergid, and its cytoplasm very diffuse. Antipodals represent extreme of preservation for this stage. Upper antipodal drawn in from second section.

first meiotic division before the rim of the outer integument passes beyond that of the inner (figs. 16, 28). The axis of the spindle for this division in the cases observed is parallel to the long axis of the ovule (fig. 24). Of the resulting cells the chalazal one is usually slightly larger (fig. 30). Division of the two daughter nuclei follows quickly. In two of the four instances observed there is a slight lag in the division of the micropylar nucleus, while in the others division is simultaneous. In all the spindles the axes were either oblique or across the cells and at various angles with each other. Walls were not formed, nor was there indication of a cell plate. The result is four megaspore nuclei in two cells,—the chalazal one, as subsequent events show, being the beginning of the 2-nucleate phase of the megagametophyte (fig. 31).

**MEGAGAMETOPHYTE.**—Marked size differences between the two pairs of nuclei become apparent quickly, since the micropylar nuclei do not grow following division, while the chalazal pair does (fig. 31). Growth of the megagametophyte begins soon after disappearance of the spindles, and in its first phase is entirely at the expense of the micropylar cell and the two megaspore nuclei it contains, so that at the end of this phase the megagametophyte has attained the size and shape of the megaspore mother cell at its initial division (figs. 30–32). The nuclei in the micropylar cell do not change in their staining reactions until at, or just before, their obvious structural degeneration begins. When this occurs the protoplast shrinks, with the cytoplasm clinging to the wall at several points. As the cell is further crushed, the nuclei become somewhat smaller. Then the cell and its contents are reduced to a dark staining cap and finally completely resorbed (figs. 32, 33).

The two nuclei of the megagametophyte lie close together almost as soon as formed, one above the other, at or near the center of the cell. Vacuoles appear above and below the nuclei without disturbing their position (figs. 31, 32). With complete disappearance of the micropylar cell, the nuclei frequently are shifted to a side-by-side position, but in the majority of instances the two vacuoles persist. When there is only one vacuole it may occupy either end of the megagametophyte (fig. 33). The megagametophyte grows steadily at the expense of the nucellus, until it has become about double the dimensions of the megaspore mother cell. The nuclei then move apart and take up positions nearer the poles, with a vacuole between them (fig. 34). Division follows quickly, and in the three instances observed the angles of the spindle axes with the axis of the ovule and with each other vary (fig. 35).

The 4-nucleate phase of the megagametophyte is relatively brief (fig. 36). In some inflorescences the chalazal end of the megagametophytes extends down nearly to the midpoint of the nucellus, while in others it reaches no farther than a fourth or a third the nucellar length. The four megagametophyte nuclei are in two

pairs, each pair imbedded in a dense, dome-shaped mass of cytoplasm at opposite ends of a large central vacuole. When first formed all four nuclei are equal in size, but while there is considerable increase in the diameters of the micropylar pair the chalazal pair does not grow (fig. 36). Division is simultaneous in the three examples observed; and in one 4-nucleate megagametophyte whose nuclei were just about to pass into the metaphase and in three very young 8-nucleate megagametophytes, all the nuclei are in the same stage. The 8-nucleate phase at first reflects the situation of the 4-nucleate phase regarding the size differences of the nuclei and the rather dense and restricted cytoplasmic masses at the poles. Each nucleus quickly organizes its mass of cytoplasm, in which there begins a characteristic type of differentiation. In the chalazal group the cytoplasm often becomes located chiefly at the sides of the nucleus and is somewhat strung out. The chalazal polar nucleus becomes readily distinguishable when the three antipodal nuclei shrink and are more chromatic, while it at first shows little change in size or appearance. Since degeneration of the antipodals is sometimes delayed or is not simultaneous, there are instances in which the chalazal polar nucleus cannot be definitely identified for a time.

The four micropylar nuclei are easily distinguished by the time the chalazal polar nucleus can be recognized with certainty. The polar nucleus derived from the micropylar end is always slightly lower down in the group relative to the vertical axis of the megagametophyte, but it is never quite separated from the others. The positions of the egg and synergid nuclei relative to the longitudinal axis of the megagametophyte are variable, but the synergid nuclei are readily recognized by their large and rather dense masses of cytoplasm, which occupy—or shortly come to occupy—the major portion of the micropylar end of the megagametophyte and become less dense or vacuolate at the chalazal margins. The egg nucleus, crowded against the side, has a much smaller mass of cytoplasm in which a very large vacuole usually develops, either laterally or toward the micropyle. Its nucleole is usually appreciably smaller than those of the synergid nuclei (fig. 37).

Before the polar nuclei fuse the chalazal one has increased in size to equal that of the micropylar (fig. 37). Fusion always occurs in the chalazal half of the megagametophyte, usually rather close to the antipodals. The endosperm nucleus is large and sometimes migrates to the micropylar end of the megagametophyte (fig. 38). By this time, the egg apparatus is well differentiated. The antipodals have usually degenerated considerably; one or more of them may have disappeared completely. Figure 38 illustrates the extreme of preservation of antipodals at this stage; more often the cytoplasm of those persisting has collapsed against the chalazal end of the megagametophyte, and the nuclear outlines—or even the nucleoles—are difficult or impossible to distinguish. Fertilization has not been observed.



### Summary

1. The flower of *Anemopsis californica*, subtended by a small floral bract, consists of six stamens, three carpels, and an ovary completely buried in the inflorescence axis.
2. The vascular supply of the individual flower and its bract originates in a single bundle of the ring of bundles in the inflorescence axis. This bundle splits up into seven: the bract bundle and three stamen bundles alternating with three placental ones.
3. Above the ovary the stamen and placental bundles branch and anastomose in such a way as to provide one bundle for each stamen and three for each style.
4. The ovule is orthotropous, with two integuments and an eventually massive nucellus. The outer integument is initiated first. Both integuments exhibit certain differentiations in their cell layers.
5. The megaspore mother cell, which with two parietals arises from a hypodermal cell, divides twice, but a wall is formed only after the first division.
6. The pair of megaspores in the upper cell degenerate and the megametophyte is formed after two more nuclear divisions of the two megaspores in the lower cell.
7. The polar nuclei fuse in the chalazal end of the megagametophyte before fertilization.
8. The antipodals have usually disappeared or are degenerating before fertilization.

The subject of this investigation was suggested by Dr. G. M. SMITH of Stanford University. Encouragement and direction were given during the progress of the work by members of the department of botany of the University of Chicago.

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# REINVESTIGATION OF POLYSOMATY IN SPINACIA

CHARLES A. BERGER

(WITH EIGHTEEN FIGURES)

## Introduction

Root tips of *Spinacia oleracea* and some other plants contain, in addition to diploid cells with the  $2n$  number of chromosomes, many polyploid cells with  $4n$ ,  $8n$ , and rarely  $16n$  chromosomes. This phenomenon, termed polysomaty by LANGLET (13), is not the result of artificial treatment of any kind but is a regular and constantly occurring process of development. The polyploid metaphases are of two kinds (figs. 11-16), some having their chromosomes in closely associated pairs, others with unpaired chromosomes. Since its discovery by STOMPS (20) in 1910, a number of papers (14, 13, 21, 22, 16) have appeared describing the phenomenon in *Spinacia* and attempting to account for the origin of the polyploid cells. The phenomenon has been reported in other plants also (15, 13, 4, 5, 17, 11, 12, 6).

Considerable difference of opinion exists, not only regarding the origin of polysomaty but also in the description of the phenomenon itself. In the larval ileum of *Culex pipiens* and other mosquitoes, a similar developmental process occurs involving the formation of cells of different degrees of polyploidy in the same tissue. Here it has been shown (1, 2) that the polyploid condition, ranging from  $2n$  to  $64n$ , is the result of successive reproductions of the chromosomes within the resting nucleus. Two years ago a reinvestigation of the situation in *Spinacia* was begun to ascertain whether the polyploid cells may not arise in the same manner as those of *Culex*, by chromosome reproduction within the resting nucleus. At that time no one had yet proposed this solution, previous investigators having suggested that polysomaty was due to nuclear fusion or a double prophase "splitting" of chromosomes. When the present investigation had been in progress for more than a year, two workers in Sweden (7) published convincing evidence that the polyploid condition was indeed due to successive chromosome reproduction in the resting stage. They also pointed out the similarity of the process to that reported in *Culex*. Their paper and the earlier publication of LORZ (16) summarize the preceding work, and each adds valuable contributions to the discussion. Accordingly the present paper will be restricted to controversial points on which new evidence is available.

## Material and methods

Seeds of *Spinacia oleracea*, variety Old Dominion, were obtained from Dr. F. S. HOLMES of the seed laboratory of the University of Maryland Agricultural Experi-

ment Station. They were germinated on filter paper in a moist chamber. At the time the tips were removed the total length of the root was measured and recorded. The Crai fixative was used, a modification by RANDOLF (19) of the chromo-acetic-formalin mixture. For nearly all purposes of this investigation only cross sections were of value. These were cut at  $10\mu$ , and all the sections of the first 1.5 mm. were mounted so that it was possible to determine position easily by counting the sections. The preparations were stained with iodine gentian-violet orange G.

### Observations

#### NEW EVIDENCE ON DISPUTED POINTS

There is some diversity of opinion as to the tissues in which polysomaty occurs. All workers have found polyploid cells in the periblem of root tips, and some report it from other tissues as well. In *Cannabis sativa* four investigators give different results. LANGLET (13) found polysomaty in the periblem, plerome, dermatogen, and calyptrogen; DE LITARDIÈRE (15) and MEURMAN (17) found it mostly in the periblem with a few polyploid cells in the plerome; BRESLAWETZ (5) found it exclusively in the periblem. In the case of *Spinacia* there is more agreement. LORZ (16) found many polyploid cells in the periblem and one example in the plerome; GENTCHEFF and GUSTAFSSON (7) found them only in the periblem. In the work here reported polyploid cells were found only in the periblem. The work on *Cannabis*, and that of MEURMAN (17) on *Acer*, show that in other species of plants the phenomenon is not restricted to the periblem but may occur in other tissues of the root tip. Nor is the phenomenon restricted to root tips. ERVIN (6) found it in the stem tip and leaf primordia of *Cucumis melo* as well as in the roots. It is possible that this will be found to be true of *Spinacia* and other plants showing polysomaty in their root tips.

#### SEQUENCE OF POLYPLOID FORMS

By sequence is here meant the relative positions in the root where the different degrees of polyploidy occur. It is indicated by the distance in microns from the extreme tip of the root cap. LORZ (16) gives the following order:  $2n-4n$  paired;  $4n-8n$  paired;  $8n-16n$  paired. GENTCHEFF and GUSTAFSSON (7) differ from this on one point. In thirteen out of seventeen roots they found unpaired tetraploid metaphases before the paired, that is, nearer the tip. On this point the observations here recorded agree with those of LORZ. In only two out of sixteen root tips thoroughly studied (table 1) did unpaired tetraploid metaphases appear nearer to the tip than paired tetraploids. In one of these roots, no. 28, the number of polyploid metaphases was so small that their relative position is of little significance. The other root, no. 29, was relatively old, being 55 mm. in length, so it is reasonable to assume that the unpaired tetraploids had already undergone a division, in

which the chromosomes were paired, since the beginning of germination. The actual distances in microns as given by GENTCHEFF and GUSTAFSSON (7) are as follows (figures represent mean from the measurement of six roots): first  $2n$  metaphase in perilem at  $240\mu$ , first  $4n$  paired at  $430\mu$ , first  $4n$  unpaired at  $370\mu$ , first  $8n$  paired at  $480\mu$ , first  $8n$  unpaired at  $640\mu$ , and first  $16n$  paired at  $870\mu$ .

GENTCHEFF and GUSTAFSSON divided their roots into four groups—"the first consisting of roots just beginning to germinate, thus showing only a few divisions. The other groups depict the increasing germinating process." This realization

TABLE 1  
DISTANCE FROM TIP (IN MICRONS) OF FIRST AND LAST  
METAPHASES OF DIFFERENT TYPE\*

ROOT NO.	LENGTH (MM.)	FIRST				LAST			
		$2n$	$4n$	$4n$	$8n$	$2n$	$4n$	$4n$	$8n$
22.....	4-5	.....	.....	.....	.....	.....	.....	.....	.....
23.....	4-5	300	.....	.....	.....	410	.....	.....	.....
25.....	4-5	370	.....	.....	.....	460	.....	.....	.....
20.....	4-5	260	440	560	.....	600	440	560	.....
2.....	13	250	440	550	510	470	710	740	590
16.....	15	170	250	.....	380	480	420	.....	380
15.....	15	360	520	600	590	810	720	660	750
5.....	25	270	490	550	.....	680	590	760	.....
28.....	30	320	660	580	640	760	720	580	640
17.....	30	270	380	620	520	890	970	710	720
30.....	35	280	470	550	.....	1120	700	800	.....
27.....	41	350	.....	.....	.....	720	.....	.....	.....
26.....	54	270	.....	550	.....	570	.....	610	.....
29.....	55	340	600	490	620	990	750	820	740
31.....	80	300	530	570	790	860	550	810	790
32.....	80	300	590	630	720	720	680	720	720

\* Paired metaphase condition indicated by heavy type.

that the age of the root makes a difference in the types of divisions was an important contribution, but apparently it was not applied with sufficient precision. These workers did not actually measure the roots, nor do they give even approximate lengths to their four groups. Early in the present investigation it became evident that the age of the root made a difference in the kind of divisions found, and accordingly each root was measured at the time of fixation. Tables 1 and 2 give the lengths of the roots in millimeters and the position and kind of metaphases found in each tip. A study of embryos dissected from seeds before the beginning of germination showed that the radicle is already fully formed and differentiated at this stage. The data of tables 1 and 2 may be interpreted as follows: Young germinating roots under 4 mm. in length contain no divisions. Growth thus far is due solely to increase in cell size through intake of water. The first divisions begin in roots

4-5 mm. in length. Differentiation into the primary meristems, dermatogen, periblem, and plerome begins about  $250\mu$  from the tip. In time this differentiation takes place during embryo formation. Paired tetraploid metaphases are regularly found nearer the tip than the unpaired. Tetraploids are rarely found in roots 5 mm. long but become increasingly numerous in longer roots. Paired octoploids are first found in roots about 13 mm. in length  $510\mu$  from the tip. Figure 18 shows the distribution of metaphases of different types in the root. The fine line indicates the extreme distribution and the superimposed heavy line the average distribution. Paired metaphase classes are underlined.

TABLE 2  
NUMBER AND PERCENTAGE OF TOTAL NUMBER OF METAPHASES  
OF DIFFERENT TYPE\* IN PERIBLEM

ROOT NO.	LENGTH (MM.)	NUMBER				TOTAL	PERCENTAGE			
		<u>2n</u>	<u>4n</u>	<u>4n</u>	<u>8n</u>		<u>2n</u>	<u>4n</u>	<u>4n</u>	<u>8n</u>
22.....	4-5	.....	.....	.....	.....	.....	.....	.....	.....	.....
23.....	4-5	3	.....	.....	.....	3	100	.....	.....	.....
25.....	4-5	2	.....	.....	.....	2	100	.....	.....	.....
20.....	4-5	10	1	1	.....	12	84	8	8	.....
2.....	13	20	14	3	3	40	50	35	7.5	7.5
16.....	15	15	5	.....	1	21	71	24	5	.....
15.....	15	26	9	2	5	42	62	21	5	12
5.....	25	2	.....	.....	.....	2	100	.....	.....	.....
28.....	30	10	2	1	1	14	71	14	7.5	7.5
17.....	30	17	24	2	4	47	36	51	4	9
30.....	35	36	8	6	.....	50	72	16	12	.....
27.....	41	9	.....	.....	.....	9	100	.....	.....	.....
26.....	54	12	.....	3	.....	15	80	.....	20	.....
29.....	55	56	6	7	3	72	78	8	10	4
31.....	80	16	2	3	1	22	73	9	14	4
32.....	80	15	5	2	1	23	65	22	9	4

\* Paired metaphase condition indicated by heavy type.

#### CRITERIA OF POLYPLOIDY

The preceding data were secured by counting metaphases only; no late pro-phases or early anaphases were included. There are other criteria of the degree of polyploidy which, though less certain than metaphase counts, are still of value. There is a general agreement between cell size and degree of polyploidy, although in any particular case this norm is not infallible, since one dimension of the cell is not readily available in sections. Nuclear size is somewhat more reliable than cell size, the nucleus being less variable in shape and not subject to vacuolization. Two other useful criteria were the number and size of nucleoli and the number of heteropycnotic satellites associated with the nucleolus. Both are applicable to cells in the resting stage. The first, nucleolar size and number, was discovered by DE MOL

(18) and has been used extensively by HEITZ (8); the second, satellite number, is new to cytological study (3).

#### NUCLEOLUS

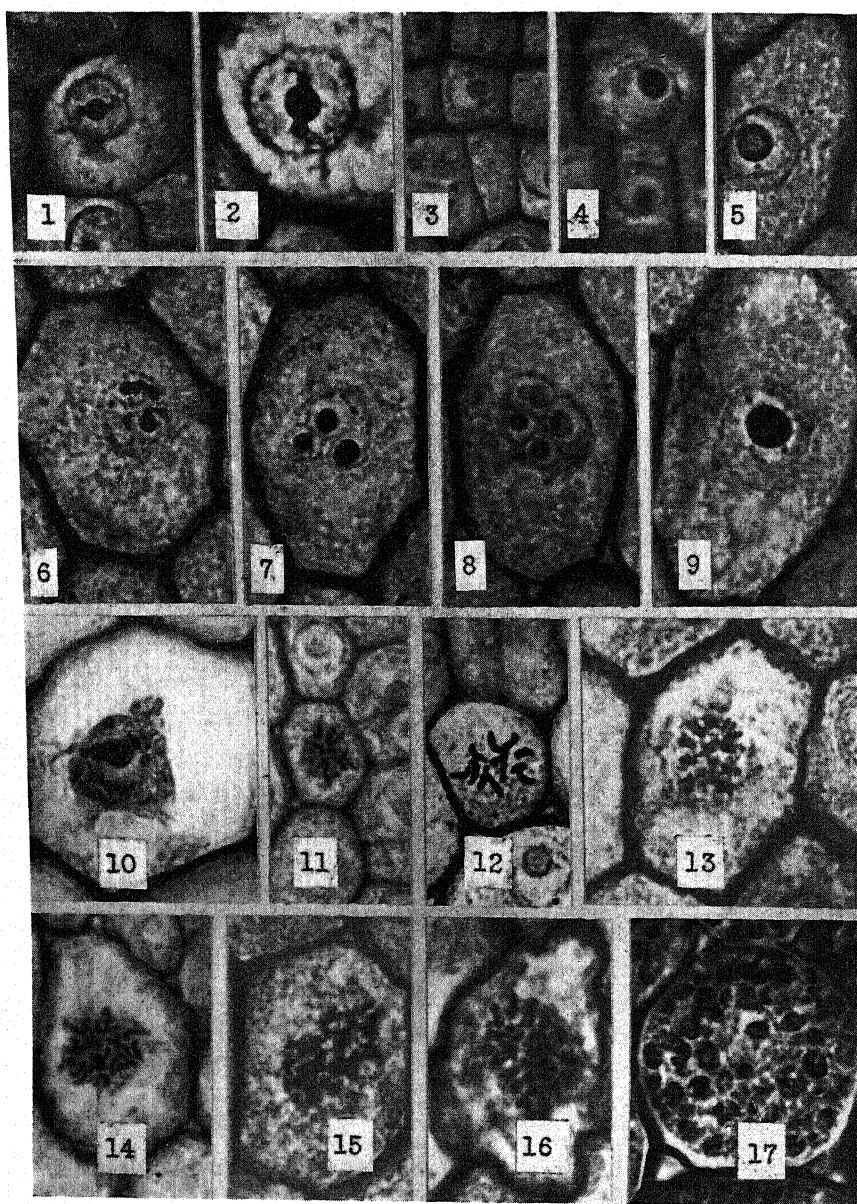
*Spinacia oleracea* has a diploid number of twelve chromosomes (figs. 11, 12). They have been described by LORZ (16) in great detail. One of the haploid set of six has a satellite attached by a short secondary constriction. The nucleolus is formed at this constriction. The regularity in the behavior of the nucleolus as worked out by HEITZ (8) in a number of plant species has been fully confirmed in this investigation. *Spinacia* is very favorable material for this study, not only because of the presence of several degrees of polyploidy but also because there is only one nucleolated or SAT-chromosome, and the situation does not become too complicated for analysis. Diploid cells always have either two small or one large nucleolus. In the region of the periblem where tetraploid cells are found, resting nuclei have four small nucleoli, one large nucleolus, or an intermediate number, in the process of fusion. The situation in the large cells of the octoploid region is similar, the nuclei containing a varying number of nucleoli ranging from eight small to one very large one. At telophase there is usually a number of small nucleoli which come into contact and fuse during the transition to resting stage. Transitional or early resting stages of this type have been interpreted by some earlier investigators as evidence for the origin of polysomaty by nuclear fusion. Figures 6-9 show transitional stages from telophase to resting stage of octoploid cells.

#### SATELLITES IN POLYPLOID CELLS

The satellite of *Spinacia* is heteropycnotic in the resting nucleus. It is either in contact with the nucleolus (figs. 1-4) or is situated near the nucleolus and joined to it by a thin thread which may be single or double (figs. 4, 5, 9, 17). The nucleolus is surrounded by a clear region. Whether this region is a fixation artifact or not is immaterial; it appears after all ordinary fixatives and is most useful in affording an unobstructed view of the satellite and its attachment thread. Diploid cells (figs. 1-4) have two satellites, tetraploid cells (fig. 5) have four, and octoploid cells (fig. 9) have eight. Older vacuolated cells which have either ceased germination divisions or have not divided since embryo formation frequently have their satellites grouped together in masses (fig. 10), from which at times threads stretch out to join the chromonemal network under the nuclear membrane. In favorable cases a count of the number of satellites or of the attachment threads is a reliable index of the degree of polyploidy of a resting nucleus.

#### ORIGIN OF POLYSOMATY

The various hypotheses proposed to account for the origin of the polyploid condition may be grouped into three classes: (1) Those explanations involving some



FIGS. 1-17.—Cells from various tissues of root tip of *Spinacia oleracea*. Distance of cell from tip given in each case. Fig. 1, diploid cell from calyptra showing nucleolus with two satellites (230  $\mu$ ). Fig. 2, same cell at higher magnification to show double chromonemata attached to each satellite (230  $\mu$ ). Fig. 3, diploid cell from calyptra; nucleolus faintly stained; satellites pycnotic (360  $\mu$ ). Fig. 4, diploid cell from perilem; nucleolus with satellites not in immediate contact (360  $\mu$ ). Fig. 5, tetraploid cell from perilem; one large nucleolus with four satellites, two of which show double structure (700  $\mu$ ). Fig. 6, octoploid cell from perilem with many nucleoli fusing; very early resting stage after  $8n$  division (700  $\mu$ ). Fig. 7, same with three nucleoli, one showing two satellites; early resting stage (700  $\mu$ ). Fig. 8, same with four nucleoli; early resting stage (660  $\mu$ ). Fig. 9, same showing single large central vacuole and has probably not undergone any divisions since embryo formation; satellites seen in two large groups, from one of which are many attachment threads (1040  $\mu$ ). Fig. 10, same in region of elongation; cell has large central vacuole and has probably not undergone any divisions since embryo formation; satellites seen in two large groups, from one of which are many attachment threads (1040  $\mu$ ). Fig. 11, diploid metaphase; outer layer of perilem (290  $\mu$ ). Fig. 12, same (410  $\mu$ ). Fig. 13, paired tetraploid metaphase from perilem (570  $\mu$ ). Fig. 14, unpaired (740  $\mu$ ). Fig. 15, paired octoploid metaphase from perilem (720  $\mu$ ). Fig. 16, same (380  $\mu$ ). Fig. 17, polyploid cell from perilem of radicle dissected from seed before germination; cell

kind of cell or nuclear fusion, either in normal or in binucleate cells or following some process of pseudo-amitosis or restitution nucleus formation. Theories of this type were proposed by STOMPS (20), BRESLAWETZ (4, 5), MEURMAN (17), HUSKINS and SMITH (9), and WULFF (22). Because of later evidence this solution must be abandoned. (2) Those solutions involving a double prophase split. This was suggested in slightly different form by DE LITARDIÈRE (14), LANGLET (13), and LORZ (16). In the light of recent findings on internal chromosome reproduction and of

later work on polysomaty in *Spinacia* this solution must also be rejected. (3) The most convincing interpretation of the origin of polysomaty is that it arises as a result of double chromosome reproduction in the resting nucleus. The credit for this solution must be given to GENTCHEFF and GUSTAFSSON (7), although, owing to the results of some X-ray work, they have compromised and place the actual reproduction at some ill-defined period between resting stage and the earliest visible prophase. These workers reject the double prophase split because in favorable cases the polyploid condition, both paired and unpaired, can be seen in earliest prophase. They also note that the paired chromosomes are relationally coiled from earliest prophase, which can mean only that they have come out of resting stage in the paired condition. On both of these points the observations here recorded are in full agreement. The paired, relationally coiled, prophase chromosomes are clear and unmistakable. At late prophase, when the pairs are contracted, they take a form described by earlier workers as somatic-diakinesis. The significance of the somatic-diakinesis figures is now clear. They are late prophase stages of paired polyploid cells. The chromosomes are considerably contracted and are held together in pairs by the remaining relational coiling, which in this way simulates chiasmata.

After subjecting dry seeds to X-radiation, GENTCHEFF and GUSTAFSSON (7) obtained in the germinating root paired and unpaired chromosome fragments but

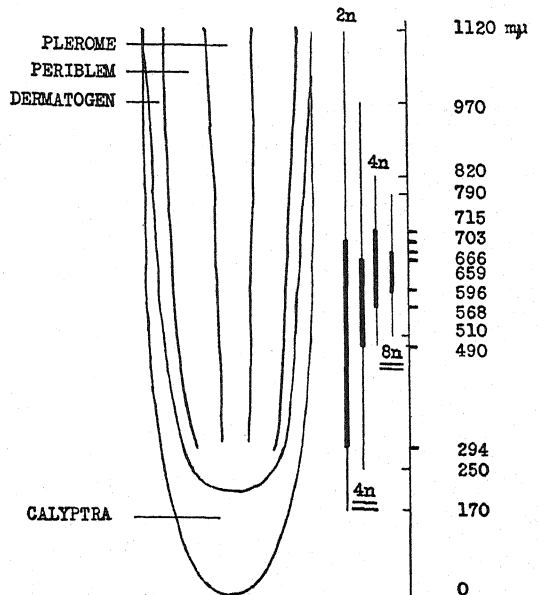


FIG. 18.—Distribution of different degrees of polyploids in root tip of *Spinacia oleracea*. Fine lines represent extreme distribution; heavy superimposed lines, average distribution. Classes twice underlined represent polyploids with paired chromosomes.



chromatid fragments. From this they concluded that the chromosomes in the resting nuclei of dry seeds are single. These results also led to their view that the actual doubling occurs at a stage between resting and earliest visible prophase. Without questioning the value of X-ray work in general, it is doubtful whether the singleness of the chromosomes was demonstrated. The cells of the dry seed prior to germination are highly dehydrated, the cytoplasm is plasmolyzed and shrunken away from the cell wall, the nucleus is likewise considerably shrunken and irregular in outline, being indented on all sides by encroaching starch grains. In such a nucleus the chromosomes also are no doubt dehydrated, and it is quite possible that the members of a reduplicated chromosome would be so close together that they would act as a single unit. At any rate until more is known about the condition of the chromosomes in the abnormal nuclei of dry seeds, the evidence for their singleness will remain inconclusive. Since the doubling has taken place before prophase, and since chromosome reproduction leading to polyploidy has been shown to take place during the resting stage in a number of insects, there seems no reasonable doubt that the polyploid condition in root tips is likewise due to a reduplication of the chromosomes in the resting nucleus while the nucleus itself is increasing in size and volume. In a recent paper KOSTOFF (10) mentions the polysomaty situation in passing and accepts the preceding explanation of its origin.

GENTCHEFF and GUSTAFSSON explain the apparent presence of unpaired before paired tetraploids by supposing that polysomaty may be established before germination begins. As already noted, it is doubtful whether the unpaired condition does actually precede the paired; nevertheless there is other evidence to show that polysomaty is established before germination. This evidence comes from applying some of the criteria of polyploidy just mentioned to the periblem cells of the radicle before germination and to the cells of the non-meristematic region of young germinating roots.

The seed of *Spinacia* contains a fully formed embryo. With the exception of vacuolization, all the differentiation present in young germinating roots is already present in the seed. Calyptra, dermatogen, periblem, and plerome are differentiated, and the cells of the periblem show the progressive increase in size characteristic of growing roots. There are minor differences, of course; all the cells of the radicle are plasmolyzed, and the cytoplasm is packed with starch grains. These granules stain deeply and obscure most of the nuclear details, yet in favorable cases the nuclei of the larger cells may be seen to have a polyploid number of satellites and attachment threads. Figure 17 shows a cell which is undoubtedly a polyploid (tetraploid or octoploid) by all the criteria which can be applied to a resting cell.

In connection with the origin of the polyploid condition, METZ (personal communication) raised the interesting question as to whether cells showing the higher

degrees of polyploidy had arrived at that condition by successive reproductions during a prolonged resting stage or after passing through at least one division in each of the lower degrees of polyploidy. Previous workers have not been clear on this point, but all appear to favor the latter interpretation; indeed none seems to have considered the first possibility. Some evidence on this question is available. In the mosquito (1, 2) high degrees of polyploidy are reached during a prolonged resting stage, and when division ensues the prophase chromosomes appear in closely associated groups of eight or sixteen sister chromatids. The condition of the satellites and attachment threads of such a cell as shown in figure 10 seems to favor a similar interpretation. This cell most probably became octoploid during embryo formation and, owing to its distal position in the radicle, did not undergo division at germination but immediately began differentiation by elongation and vacuole formation. Its multiple attachment threads and satellites are in two groups. It is possible that the polyploidy established before germination may have arisen by successive doublings of the chromosomes without the intervention of any mitosis. A thorough study of the embryology of *Spinacia* would be necessary to determine this point.

There is evidence, however, that the polyploid condition also arises after the beginning of germination during growth of the root. The paired condition and relational coiling of the chromosomes at prophase and metaphase indicate that the double chromosome reproduction has taken place during the preceding resting stage. If, therefore, the paired condition and the relational coiling are found in cells which are known to have undergone at least one mitosis since the onset of germination, this would constitute proof that the doubling had taken place since the beginning of germination. Since mitosis is confined to the first millimeter of the root tip in *Spinacia*, it seems certain that in a root several inches long all the cells of the growing point will have undergone some divisions since the beginning of germination. Roots nos. 31 and 32 in tables 1 and 2 satisfy this condition. Both were 80 mm. long when their tips were fixed, and both have many polyploid prophases and metaphases showing pairing and relational coiling.

Whatever may be the situation in polyploidy established before germination, it appears certain that polyploidy established after germination originates by a process of successive chromosome doubling, with at least one mitosis intervening at each new degree of polyploidy. This is shown by the fact that in prophases and metaphases of paired tetraploids and octoploids groups of four or eight chromosomes are never found; only pairs of sister chromosomes are observed. In an octoploid where four pairs of satellited chromosomes are present, the pairs are scattered at random. This condition is in striking contrast with that found in *Culex* and other mosquitoes, where the first division after the reduplication period shows close association of high multiples of sister chromosomes.

### Summary

1. Polysomaty in the root tip of *Spinacia* is apparently restricted to the periblem.
2. The different types of polyploid cells appear in the following sequence in regard to time of appearance and position: diploid, tetraploid with paired chromosomes, tetraploid unpaired, octoploid paired.
3. Polyploidy arises by successive reproduction of chromosomes during the resting stage of the nuclear cycle.
4. The paired condition of the chromosomes indicates that the double chromosome reproduction has taken place during the resting stage immediately preceding.
5. Polyploid cells with unpaired chromosomes have undergone at least one mitotic division since the time of double reproduction.
6. The degree of polyploidy of cells in the resting stage can be judged by the number and size of nucleoli and the number of heterochromatic satellites associated with the nucleolus.
7. Polysomaty is already established in the periblem of the radicle before the beginning of germination. Double chromosome reproduction therefore takes place during embryology. Double chromosome reproduction also takes place during growth of the root after germination has begun. This is shown by pairing and relational coiling in polyploid prophase of older roots.
8. Polyploidy established after the beginning of germination is due to successive chromosome doubling, with at least one mitosis intervening at each new degree of polyploidy.

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# RESPONSES OF VEGETATIVE PARTS OF PLANTS FOLLOWING APPLICATION OF EXTRACT OF POLLEN FROM *ZEA MAYS*

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(WITH TWELVE FIGURES)

## Introduction

The existence of growth-stimulating properties in reproductive bodies of some plants was first observed in connection with the development of fruits. As early as 1849 it was found that application of spores of *Lycopodium* to the stigmas of certain cucurbits resulted in growth and development of seedless fruits (4). Parthenocarpic development of fruit has been induced also by applying pollen of other species to the stigmas of certain cucurbits, grapes, and orchids (14, 15), whether such pollen were dead or living. More recently, extracts of pollen capable of inducing parthenocarpy have been obtained, thus indicating that the stimulus to develop is chemical in nature (3, 7, 12, 13, 23). LAIBACH and KORNMAN (12) applied extracts of orchid pollen to plant parts other than the carpels; they observed that when an extract of pollen was applied to them unilaterally, oat coleoptiles bent and elongated more rapidly when bathed in a solution of pollen extract. They also observed that epicotyls of *Phaseolus* and petioles of *Coleus* bent following unilateral applications of pollen extract. LA RUE (13) demonstrated that extracts of pollen retarded the formation of abscission layers in debladed petioles of *Coleus*. Similar responses have also been effected by applying various synthetic growth-regulating substances to the stems and fruits of some plants (1, 2, 5, 6, 19, 20, 22).

The investigations here reported deal with studies of growth responses and histological changes resulting from application of extracts of corn (*Zea mays*) pollen to different vegetative parts of plants. A detailed study was made of responses that occurred when the extract was applied to the cut surface of decapitated stems, or to the epidermis of the stems of intact plants, and these were compared with those that resulted when certain synthetic growth-regulating substances were applied in a similar manner.

## Methods

The pollen used in these experiments was collected mainly from a single cross of two varieties of yellow field corn (KYS  $\times$  US7), in July, 1940, although similar work was done in the autumn of 1939 also. Tassels having anthers about to de-

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hiscs were covered with paper bags and the pollen collected as it was shed. Anthers mixed with the pollen during its collection were separated by screening and discarded. The pollen was dried at 80° C. in a well-ventilated oven and stored in airtight glass containers, this method proving suitable, as illustrated by the fact that material thus stored for about one year still yields quantities of active extract.

Extractions were made by adding ether to pollen in proportions of 20 ml. to 5 gm. of pollen and allowing the mixture to stand over night at room temperature. The mixture was then filtered and the filtrate collected in a weighed evaporating dish. The ether was finally evaporated over a water bath at 50–60° C. until the residue attained a constant weight. A fatty residue weighing 25–35 mg., and having a melting point of 46–48° C., was obtained in this way.

With the exception of a few experiments, Baker's analyzed ethyl ether was used as a solvent with which the growth-regulating substance was extracted from the pollen (the number of such substances extracted is not known, but for simplicity the active fraction of the extracts is referred to as growth-regulating substance). The ether was not purified as suggested by VAN OVERBEEK (21), since no difficulty was experienced in obtaining highly active extracts without taking this precaution.

For use in experimental work, anhydrous lanolin was thoroughly mixed with the fatty residue remaining after evaporation of the ether, generally in proportions of one part by weight of extract to four parts of lanolin. This mixture was then applied by gently rubbing a small amount carefully on the surface of a plant so as to insure contact but to avoid rupturing of cells or cuticular layers.

For part of the histological studies, approximately 10–15 mg. of the paste was applied to the cut surfaces of decapitated second internodes in the same manner as already described in connection with similar studies (9). In other experiments it was applied to the surface of intact first internodes of non-decapitated bean plants, and to some other plants, as detailed later. In the case of bean, young vigorous seedlings were used, the applications being made when the first internode was approximately 15 mm. long, the heart-shaped leaves not fully developed, nor the second internode elongated. The stems were preserved in Navashin's solution, then sectioned with the aid of a freezing microtome, or imbedded in paraffin and sectioned in the usual manner.

In determining the size of cells it was not feasible to measure those of tissues made up of relatively few or small cells. The measurements as recorded are based on parenchymatous cells of the cortex and pith. The average number and size of such cells was determined by first selecting internodes of average length. From each of these internodes, segments 2 mm. long were removed at 5-mm. intervals and sectioned longitudinally. In one median section taken at each 5-mm. level an approximate row of cells extending from one side of the section to the other

was selected for measurement. Thus it was possible to compute the average width and length of cells of these tissues at regular intervals along the internode and to determine the dimensions of average cells for a given tissue.

**EXTRACTION AND SOLVENTS.**—The solubility of the active substance was tested by adding 40 ml. of ethyl ether, chloroform, acetone, or absolute alcohol, to 10-gm. lots of pollen and allowing the mixtures to stand at room temperature over night. The filtrates, with the exception of the aqueous ones, were evaporated to dryness on a water bath. The waxy residues were mixed with four times their weight of lanolin, and finally 50–75-mg. aliquots of these mixtures were applied unilaterally to the stems of bean seedlings. Filtrates of water extracts were evaporated at reduced pressure at 60° C. to a volume of 1–2 ml., and the liquid then mixed with 1 gm. of lanolin. As in the case of the other residues, 50–75-mg. aliquots were applied unilaterally to stems of young bean plants. All four organic solvents gave active extracts. Extracts with hot or cold water did not contain an appreciable amount of growth-promoting substances in an active form. Ether extracts of pollen which had previously been extracted with water and dried also failed to give positive tests, indicating the possibility that the growth-stimulating substance may have been soluble but unstable in water. As indicated by increased linear growth of bean stems, ether was most effective in extracting the growth-regulating material, and this solvent was therefore used in all subsequent experiments.

A number of different kinds of oils were tested as possible media in which the active substance extracted from pollen might be applied to plants. Sixty mg. each of cod liver, soybean, olive, and peanut oils were added respectively to 20 mg. of ether extract and applied unilaterally to the hypocotyls of young bean seedlings. Marked stem curvatures were observed in all cases, while plants treated with the various oils alone showed no response.

### Gross responses

#### COMPARISON OF POLLEN EXTRACT AND SYNTHETIC GROWTH-REGULATING SUBSTANCES

The effectiveness of relatively high concentrations of various synthetic growth-regulating substances in stimulating stem elongation was compared with that of the natural growth-regulating substance extracted from pollen. Two per cent lanolin mixtures of various growth substances were applied as a band approximately 2 mm. wide around the stem midway between the first and second nodes of young bean seedlings.

One week following treatment, when elongation of the first internode had practically ceased, those internodes to which pollen extract had been applied were significantly longer than controls, by approximately 43 per cent (table 1; fig. 1).

Earlier during the experiment internodes treated with pollen extract were more than twice the length of comparable controls, but subsequent growth by the controls resulted in a relative decrease of this difference when the internodes had ceased to elongate. There was a significant increase in rate of elongation during the first few days following treatment with indoleacetic acid, but subsequently the internodes to which this substance was applied ceased to elongate, formed tumors, and were at maturity 20 per cent shorter than controls.

Phenylacetic acid, naphthaleneacetic acid, and naphthalene acetamide mixtures resulted in significant retardation of stem elongation, this response being associ-

TABLE 1

EFFECT OF COMPOUNDS ON INTERNODAL ELONGATION WHEN APPLIED AS BAND 2 MM. WIDE AROUND MIDDLE OF FIRST INTERNODES OF YOUNG KIDNEY BEAN PLANTS. FIGURES REPRESENT AVERAGE LENGTH IN MILLIMETERS OF FIRST INTERNODES OF TWENTY PLANTS

HOURS AFTER TREATMENT	TREATMENT					
	CONTROL	POLLEN EXTRACT	INDOLE- ACETIC ACID	PHENYL- ACETIC ACID	NAPHTHA- LENEACETIC ACID	NAPHTHA- LENE ACETAMIDE
52.....	12.9	26.2*	15.6*	13.3	13.2	13.4
100.....	25.9	39.8*	20.6†	20.7†	17.6†	21.6†
172.....	28.5	40.8*	22.7†	23.2†	17.9†	24.4†

\* Significantly longer than control of same date; ratio 19:1.

† Significantly shorter than control of same date; ratio 19:1.

ated with tissue proliferation and root formation in the case of the acids (8) and lignification and increased cambial activity in the case of the amide (10).

It is known that the application of small amounts of indoleacetic acid may result in cell elongation, as indicated by curvatures induced in *Avena* coleoptiles and the stems of succulent plants (2, 22). A comparison was made between the amount of stem elongation which resulted from the application of small amounts of indoleacetic acid and that resulting from the use of pollen extract. A 0.002 per cent lanolin mixture of indoleacetic acid was used, as previous experiments had shown that this concentration caused the greatest amount of stem elongation of any concentration tested between 0.00002 and 2.0 per cent. Internodes treated with either pollen extract or the acid were significantly longer than comparable controls treated with lanolin alone, even as early as 24 hours after treatment. Although both the extract and the acid appreciably stimulated stem elongation, the responses were unlike in two ways. First, indoleacetic acid stimulated growth in length of stems for a relatively short time, while those treated with pollen extract continued to elongate for a longer period following treatment. Second, growth



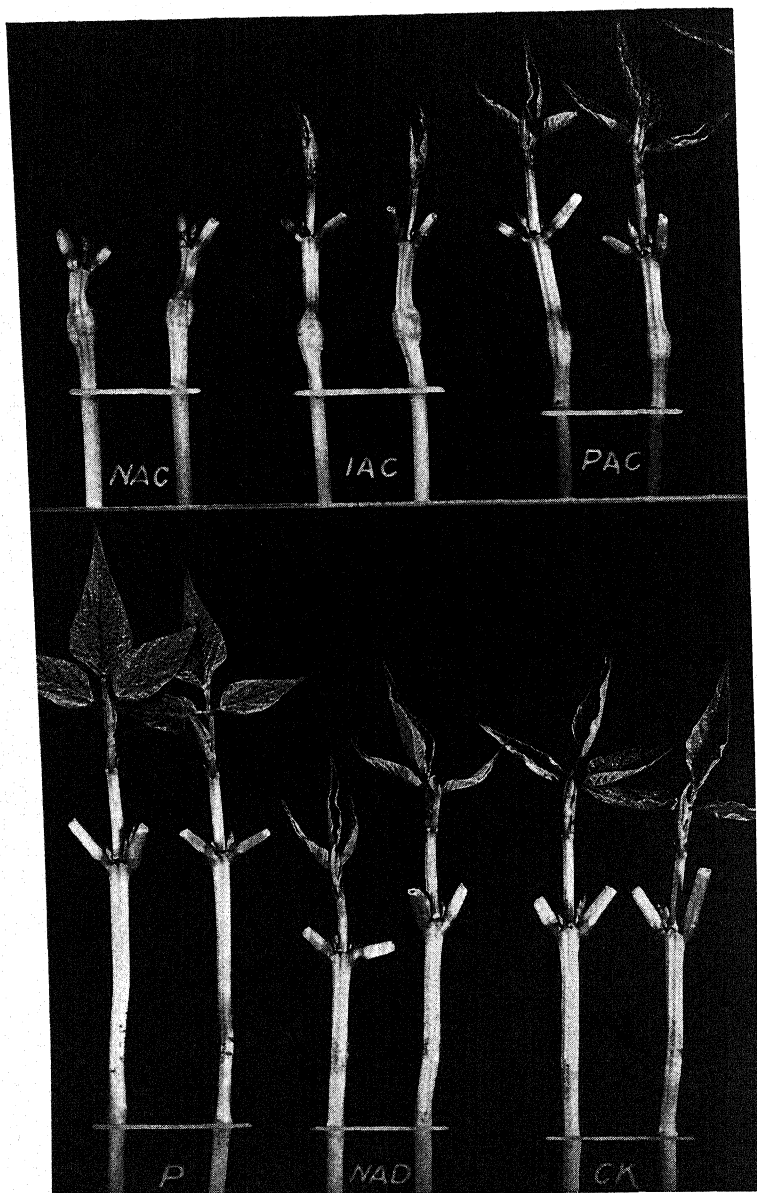


FIG. 1.—Growth responses resulting from application of 2 per cent lanolin mixtures of growth-regulating substances to first internodes of bean plants, as compared with that resulting from use of pollen extract or lanolin alone. Top: alpha naphthaleneacetic acid, beta indole-3-acetic acid, phenylacetic acid. Below: pollen extract, alpha naphthalene acetamide, and check.

stimulation as the result of application of the extract was approximately 2.6 times the maximum amount resulting from application of indoleacetic acid (fig. 2).

Still further tests were carried out to determine the effectiveness of pollen extract in stimulating linear growth. Only slight curvatures resulted when pollen extracts were applied to oat coleoptiles. Lanolin containing pollen extract in the ratio of 4:1 was applied unilaterally at one point on the upper third of the coleoptile. Somewhat stronger curvatures resulted when the extract was smeared along one side of the coleoptile for 1 cm. or more.

The application of pollen extract to the stems of different varieties of plants accelerated their rate of elongation. Plants tested included radish, flax, potato, kidney bean, soybean, marigold, tomato, *Mirabilis*, and buckwheat.

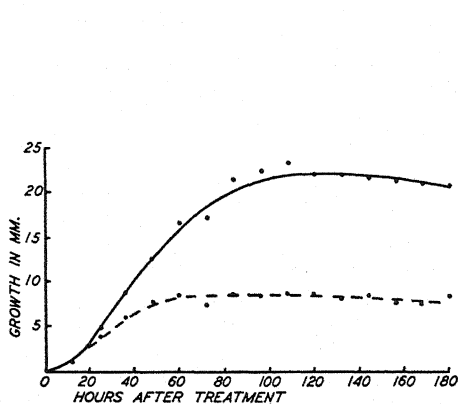


FIG. 2

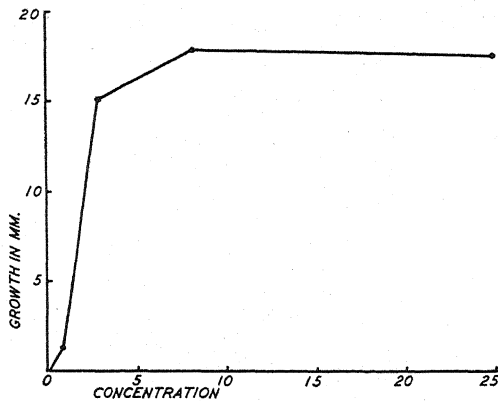


FIG. 3

FIGS. 2, 3.—Fig. 2, growth of first internodes of bean plants treated with pollen extract-lanolin mixture, compared with that of others treated with 0.002 per cent mixture of indoleacetic acid in lanolin (broken line). Values represent gain over that of controls treated with lanolin. Fig. 3, linear growth of first internodes of bean plants following treatment with different amounts of pollen extract. Values represent gain in length over that of controls during 48-hour period following treatment.

#### EFFECTS OF VARIOUS AMOUNTS OF POLLEN EXTRACT ON LINEAR GROWTH

To establish a quantitative relationship between the amount of extract applied to stems and their rate of elongation, bean plants were grown from seeds under controlled environmental conditions (17). The temperature of the room was 73–75° F., the relative humidity 65–85 per cent. Illumination was supplied for a period of 14 hours daily by means of a 60-ampere carbon arc burning Sunshine carbons and a number of 200-watt incandescent lamps evenly distributed approximately 3 feet above the plants, which together supplied an intensity of 1200–1400 foot candles.

Four concentrations of extract were made by first adding 800 mg. of lanolin

to 200 mg. of extract. This mixture was designated a 25 per cent concentration. Subsequent dilutions were made by adding 240, 960, and 3120 mg. of lanolin to 150-mg. aliquots, respectively, of the 25 per cent mixture. When the first internodes of a number of selected seedlings were approximately 15 mm. long, the plants were divided into five groups, each containing twenty-four plants. Each group was then treated by carefully applying 15 mg. of one of the four concentrations in a thin layer extending around the stem as a band 1 cm. wide. The fifth group was treated with lanolin alone as the control.

The effect of these various amounts of extract on the rate of internodal elongation was measured at intervals during the 3 days following treatment. Maximum stimulation resulted when the mixture contained 10 per cent of crude extract (fig. 3). Further dilution resulted in a marked decrease in the amount of internodal elongation. It was concluded that bean seedlings, grown under these conditions, could be used as test subjects for quantitatively measuring different amounts of substances extracted from corn pollen, which stimulate linear growth.

#### MOBILIZATION EFFECTS OF POLLEN EXTRACT

It was previously found that stems of kidney bean formed tumors as a result of tissue proliferation following applications of 2 per cent lanolin mixtures of indoleacetic or naphthaleneacetic acids (18, 19), and that this response was associated with the rapid movement of some organic substances toward the treated region. As a result of this mobilization of materials, treated portions of stems gained in dry weight more rapidly than did comparable portions of untreated stems.

It was observed that mobilization was also associated with an increased rate of stem elongation as induced by application of the pollen extract. To demonstrate this effect, a band of pollen extract-lanolin mixture was applied to the first internodes of Biloxi soybean seedlings when the internodes were approximately 15 mm. long. After the plants had been grown for 18 days in a greenhouse, the treated internodes averaged approximately 53 per cent longer and 54 per cent heavier in fresh weight than did comparable parts of controls treated with lanolin alone. Growth of the stem and leaves above the treated internode was significantly retarded, the area of the primary leaves being approximately 22 per cent less than that of controls at the end of the experiment.

In a subsequent experiment the effect of pollen extract on the accumulation of dry weight in the stems of kidney bean plants was determined. The plants were treated by placing a band of the pollen extract-lanolin mixture around the first internode when these were approximately 15 mm. long. Three days following treatment, the internodes to which the extract had been applied had gained 65 per cent more in length, 86 per cent more in fresh weight, and 81 per cent more in dry weight than had the first internodes of comparable controls treated with lanolin

alone. It was concluded that stem elongation resulting from application of the pollen extract was associated with movement of materials toward the treated region.

#### EFFECTS OF LIGHT ON ELONGATION

It is known that in certain plants internodal elongation is limited to some extent by the quality and intensity of light with which they are illuminated (16). A number of experiments were carried out to determine the effect of light intensity and quality on the response of bean internodes following the application of pollen extract.

Bean seedlings were grown under controlled environmental conditions (17). Some of the plants were supplied with a light intensity of 200 foot candles, others

TABLE 2

EFFECT OF LIGHT INTENSITY ON STEM ELONGATION FOLLOWING TREATMENT WITH POLLEN EXTRACT-LANOLIN MIXTURE. FIGURES REPRESENT AVERAGE LENGTH IN MILLIMETERS OF TWELVE PLANTS

HOURS AFTER TREATMENT	TREATMENT					
	1800 FOOT CANDLES			200 FOOT CANDLES		
	EXTRACT MIXTURE	LANOLIN	DIFFER- ENCE	EXTRACT MIXTURE	LANOLIN	DIFFER- ENCE
48. ....	48.2*	29.7	18.5†	48.8*	39.8	9.0
72. ....	56.4*	38.8	17.6†	59.7*	49.4	10.3
96. ....	59.8*	45.0	14.8†	63.6*	54.0	9.6

\* Significantly longer than control of same date grown under same light intensity; 19:1.

† Significantly greater than difference at intensity of 200 foot candles; 19:1.

with an intensity of 1800 foot candles, of light from a carbon arc. Greatest increase in linear growth over that of controls resulted from application of the pollen extract to the internodes of plants grown at the higher intensity (table 2). In other experiments even greater increase resulted when treated plants were grown in natural daylight of a relatively high intensity, while little or no increase over controls was observed when the plants were grown in complete darkness.

The quality of light was varied, under controlled conditions, by illuminating a group of plants with light from incandescent lamps which supplied an intensity of 700 foot candles at the leaf surface, while another group was illuminated with approximately the same intensity of light supplied by means of Daylight fluorescent tubes. Plants grown under the fluorescent tubes produced relatively short stems, while those grown under the incandescent lamps developed elongated internodes. Internodal elongation resulted following the application of pollen extract only in the case of those plants illuminated by means of fluorescent tubes (table 3).

In summary, it was observed that the greatest increase in linear growth resulted when the extract was applied to internodes of plants grown in light of an intensity and quality most favorable for the development of short sturdy stems, and there was little or no difference between internodal elongation of treated and control plants grown under light conditions that favored etiolation.

#### EFFECTS OF POLLEN EXTRACT ON STRUCTURES OTHER THAN FIRST INTERNODE

To determine the effect of pollen extract on the growth of vegetative parts of bean plants other than the first internode, buds, leaf tissue, second and third internodes, and roots were treated. Terminal vegetative buds approximately 3 mm.

TABLE 3  
EFFECT OF LIGHT QUALITY ON INTERNODAL ELONGATION FOLLOWING TREATMENT  
WITH POLLEN EXTRACT. FIGURES REPRESENT AVERAGE LENGTH IN MILLI-  
METERS OF FIRST INTERNODES OF THIRTY-SIX PLANTS

HOURS AFTER TREATMENT	TREATMENT					
	FLUORESCENT LIGHT			MAZDA LIGHT		
	EXTRACT MIXTURE	LANOLIN	DIFFER- ENCE	EXTRACT MIXTURE	LANOLIN	DIFFER- ENCE
48.....	31.6*	13.9	17.7	35.3†	46.7	11.4
96.....	48.9*	25.3	23.6	61.2†	64.5	3.3
144.....	50.1*	31.5	18.6	68.5	68.7	0.2

\* Significantly longer than control of same date grown in same light quality; 19:1.

† Significantly shorter than control of same date grown in same light quality; 19:1.

long, and others 1 cm. long, were coated with a thin layer of melted pollen extract-lanolin mixture. Individual measurements made at repeated intervals following treatment showed no significant increase in either the length or the fresh weight of treated as compared with untreated buds. When the mixture was applied as a 5-mm. strip across the lower surface of young bean leaves, cell enlargement was greatly stimulated near the treated region, causing the leaves to bend at this point and curl upward and backward so that the distal end rested against the petiole end. Similar treatments applied to the upper surfaces of leaves failed to cause noticeable curvatures of the blades.

Elongation of second and third internodes of kidney bean plants was increased as a result of the application of the extract-lanolin mixture.

To test the effect of the extract on elongation of roots, kidney bean seeds were germinated in sand. When their tap roots were approximately 25 mm. long the plants were carefully removed from the sand, 120 uniform plants selected, and di-

vided into six lots of twenty plants each. The tap root of each plant was measured, and then the plants in three of these groups were treated by applying a band 3 mm. wide of the extract-lanolin mixture around each tap root at the region of elongation. A comparable number of plants were treated in the same way with lanolin to which no extract was added, as controls. The seedlings were kept moist, and each group was replanted individually in sand immediately following treatment. Twenty-four hours after treatment the plants were again removed from the sand and the tap roots measured. None of the roots showed signs of injury. Small tumors, approximately twice the diameter of the root, developed immediately distal to the treated region to which the extract-lanolin mixture was applied. The average increase in the length of roots treated with the mixture was 24.6 mm., that of controls 27.3 mm., a difference statistically significant at the 5 per cent level, indicating that applications of pollen extract-lanolin resulted in the inhibition of root elongation.

### Histological responses

#### LATERAL APPLICATIONS

Histological examinations showed that the main effect of lateral applications of pollen extract to first internodes resulted in an increase in the length of the

TABLE 4

AVERAGE LENGTH, WIDTH, AND NUMBER OF PITH CELLS IN FIRST INTERNODES OF BEAN STEMS TREATED WITH LANOLIN COMPARED WITH LENGTH, WIDTH, AND NUMBER OF OTHERS TREATED WITH POLLEN EXTRACT-LANOLIN MIXTURE. LENGTH AND WIDTH EXPRESSED IN MICRONS (SEE METHODS)

INTERNODE	TREATMENT					
	LANOLIN			EXTRACT MIXTURE		
	NO. OF CELLS PER INTERNODE	AVERAGE LENGTH OF CELLS	AVERAGE WIDTH OF CELLS	NO. OF CELLS PER INTERNODE	AVERAGE LENGTH OF CELLS	AVERAGE WIDTH OF CELLS
I.....	824	6.5	5.8	707	14.5	5.4
II.....	745	7.3	6.4	778	13.7	6.1
III.....	963	6.3	5.8	749	13.6	5.9
IV.....	761	7.1	6.0	796	13.2	5.7
V.....	753	7.6	5.7	790	13.1	5.8

cells of various stem tissues. The average length of pith cells of internodes treated with extract was approximately 94 per cent greater than that of pith cells of comparable controls (table 4; fig. 5). The cells having the greatest average length, 246 per cent greater than that of controls, occurred in that portion of the pith directly beneath the surface to which the extract was applied (fig. 4). Pith cells proximal or distal to this treated portion were less affected; and at the ends of the

internode, approximately 1 cm. away from the treated region, their length was only slightly greater than that of controls. Measurements showed that the growth in length and width of cortical cells was stimulated by the application of extract, particularly near the treated region. No abnormal twisting or tearing of tissues could be observed which indicated that tissues other than pith and cortex elongated correspondingly. It was evident that the increase in length of internodes as the result of treatment with pollen extract was associated mainly with increase in the length of cells in the pith and cortex, rather than with cell division. This

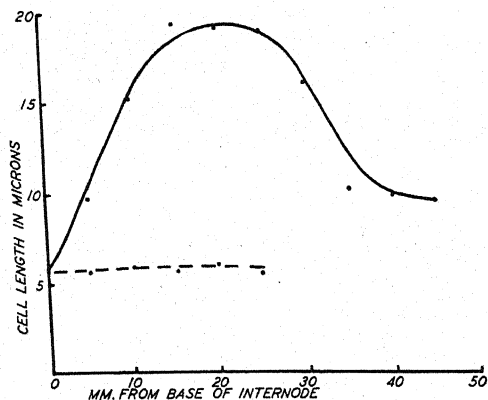


FIG. 4.—Average length of cells in pith of first internode of bean plants 96 hours after surface of internode was treated with pollen extract-lanolin mixture, compared with average length of pith cells of control treated with lanolin (broken line).

appeared to be true in other tissues, although direct measurements were not made. The increased internodal length associated with the application of a 0.002 per cent indoleacetic acid paste was also characterized by an increase in the length of the cells of various tissues.

#### APICAL APPLICATIONS

The second internodes were decapitated just as the first trifoliate leaf was expanding, the usual care being exercised in pressing the lanolin-pollen extract mixture firmly against the cut surface. The plants were maintained under usual greenhouse

conditions, and experiments were repeated at several seasons during the year. Except for slight variations in degree and extent of development of the apical tumors, the sequence of events as given here is representative.

At the end of 36 hours following treatment, the epidermal, cortical parenchyma, and endodermal cells show considerable radial elongation. For the most part the chloroplasts and starch have disappeared, the contents of the endodermal cells are more dense, and in many instances some of these have divided tangentially at least once (fig. 6). The pericyclic, phloem, and cambial cells show no apparent response.

At the end of 60 hours the epidermal cells have elongated radially still more and may have divided radially, particularly in the vicinity of an epidermal hair or a stoma. The content of the cortical parenchyma cells is very dense, and the cells have divided in all planes, the divisions being numerous just beneath the epidermis or near a stoma. The endodermal cells have undergone several divisions, both radially and tangentially, but in many instances the derivatives are not readily

distinguishable from those of the cortical parenchyma (fig. 7). In other instances the endodermal derivatives are clearly distinguishable from near-by cells, and many of them have differentiated as tracheids, especially those adjacent to the ray cells, while others continue to proliferate and form a wide band of meristematic cells

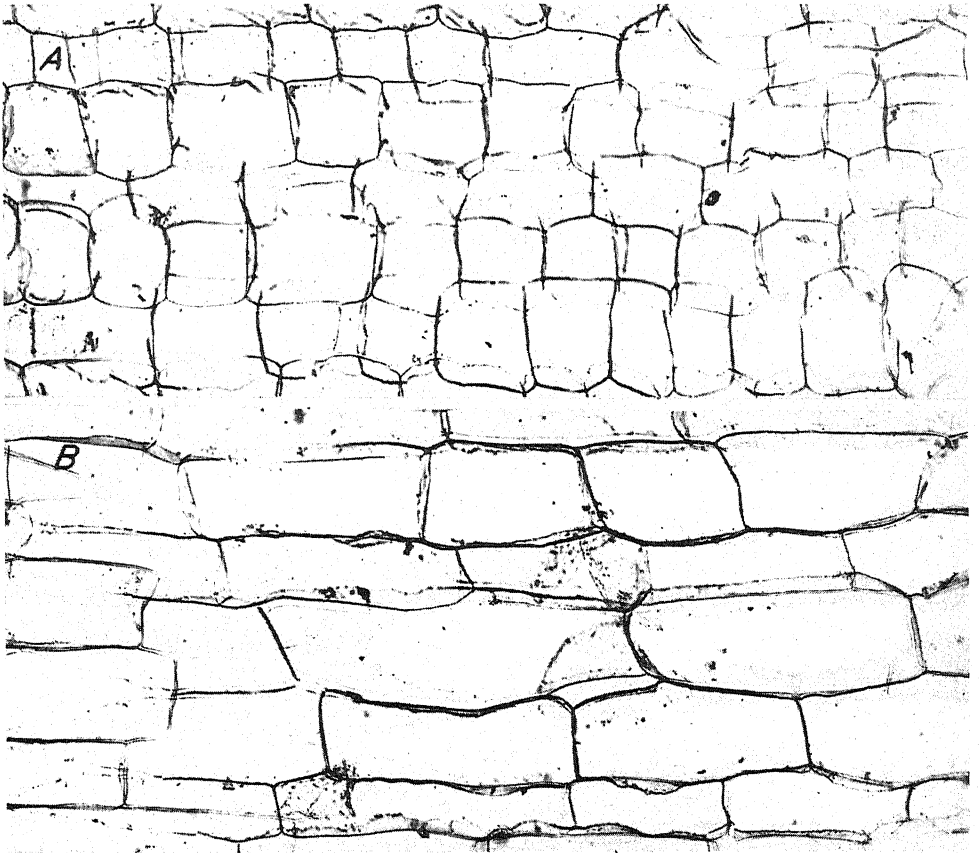


FIG. 5.—Representative pith cells of first internode of bean plants 96 hours after outer surface had been treated with ring of lanolin (A) compared with those of comparable internode treated in same way with pollen extract-lanolin mixture (B).

completely capping phloem and pericyclic cells. Some of the cortical cells are arranged as meristematic bands extending from the epidermis just beneath a stoma to the cells of the endodermis or in some instances directly to those of a ray. The ray cells themselves are but slightly meristematic, but from some of these the interfascicular cambium originates, while others divide several times and their derivatives mature as tracheids. Cells of the pith, cambium, and phloem show little or no response, other than that the former enlarge somewhat, occasionally divide,



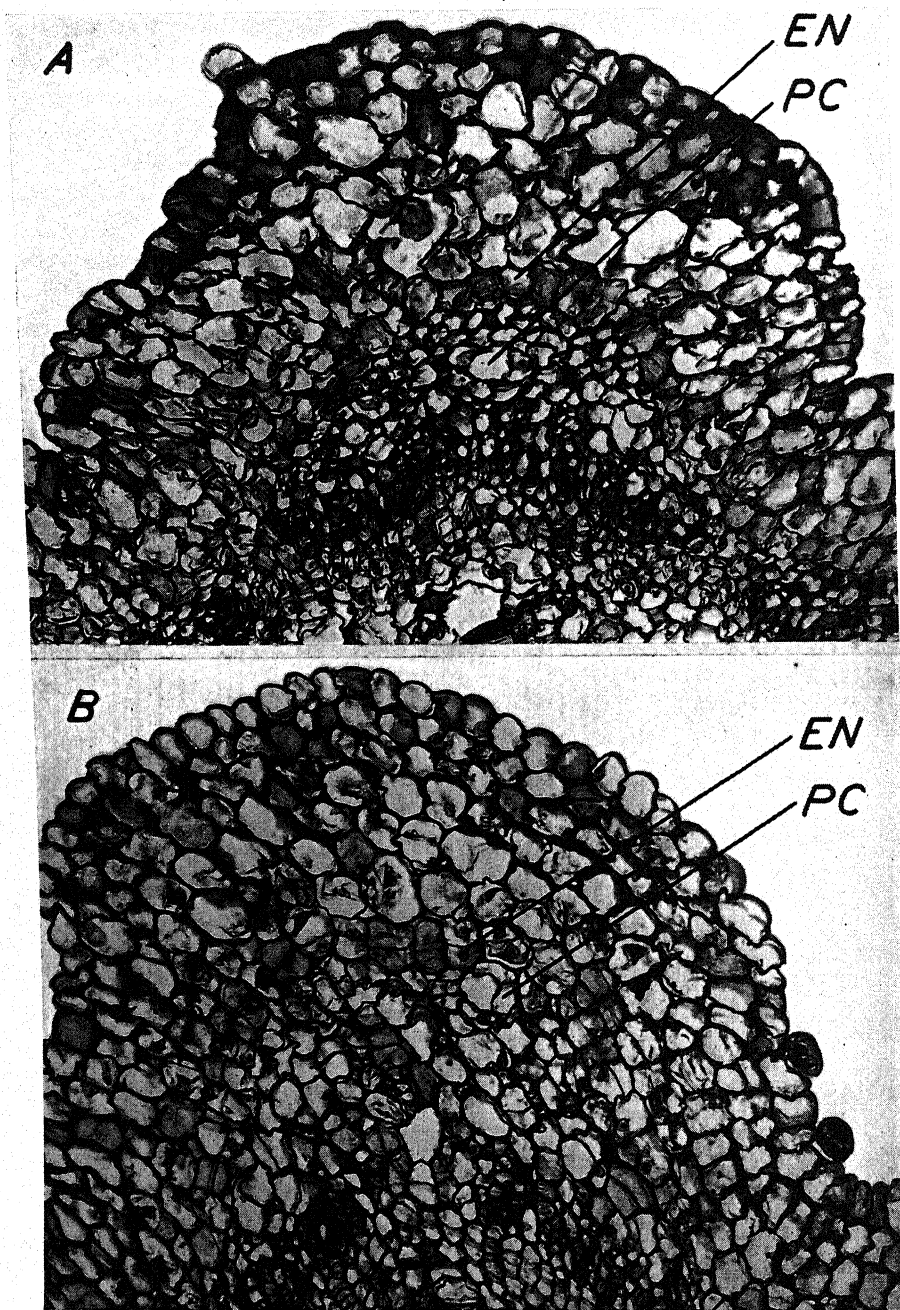


FIG. 6.—Transsections of bean stem decapitated, and cut surface treated with pollen extract-lanolin mixture, 36 hours after application. Cells of epidermis, cortex, and endodermis enlarged appreciably. *A*, endodermal cells enlarged and contents more dense; *B*, many of these have divided tangentially.

and remain more active than do similar cells of plants treated with lanolin only (fig. 8).

At the end of 86 hours the same types of change, growth, and development still continue. Many more cells have matured into tracheids, however, and this is particularly true in the case of the cells derived from the endodermis. Sieve tubes and

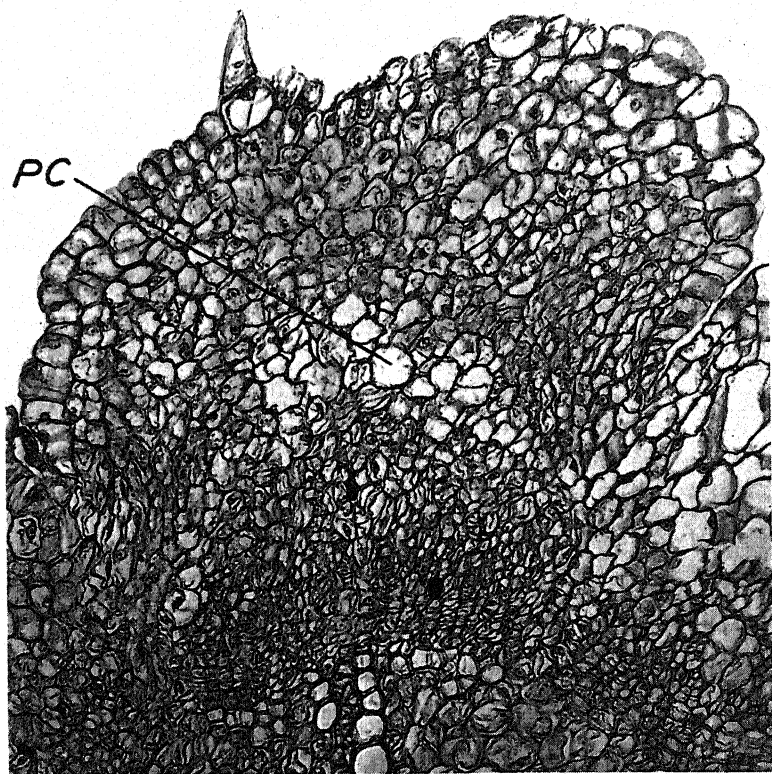


FIG. 7.—Transection near treated surface 60 hours after application. Many cortical cells have divided in various planes; pericyclic fibers have enlarged somewhat, but their walls have not increased appreciably in thickness; other tissues have remained active but show no marked meristematic activity. Proliferated endodermal cells not readily distinguishable from rest of cortex.

companion cells have also matured from derivatives of similar origin. Direct connections with the phloem and cambium of the stele made through the rays are mature and clearly delimited. The cortical parenchyma cells are active, and from the strands of meristematic cells larger numbers of scattered tracheids have matured. The vascular strands extend to a stoma. The epidermal cells continue active radial division and show no rupturing, despite the expansion of tissues beneath them (fig. 9).

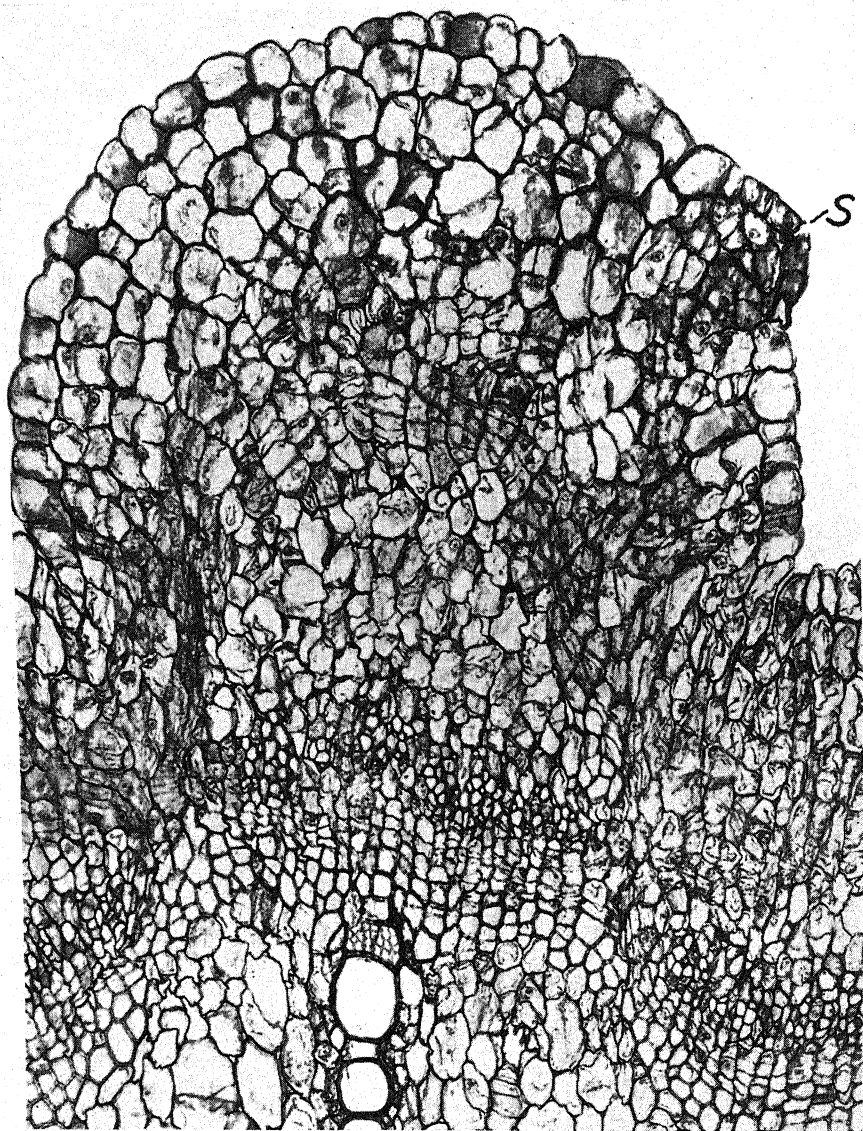


FIG. 8.—Section at about same level and at same period after treatment as fig. 7. Endodermal derivatives clearly distinguishable, some of them differentiated as tracheids near ray cells. Cortical parenchyma active, particularly the band of cells below a stoma (*S*) extending to endodermis. A few of the cells of the primary phloem have also divided.

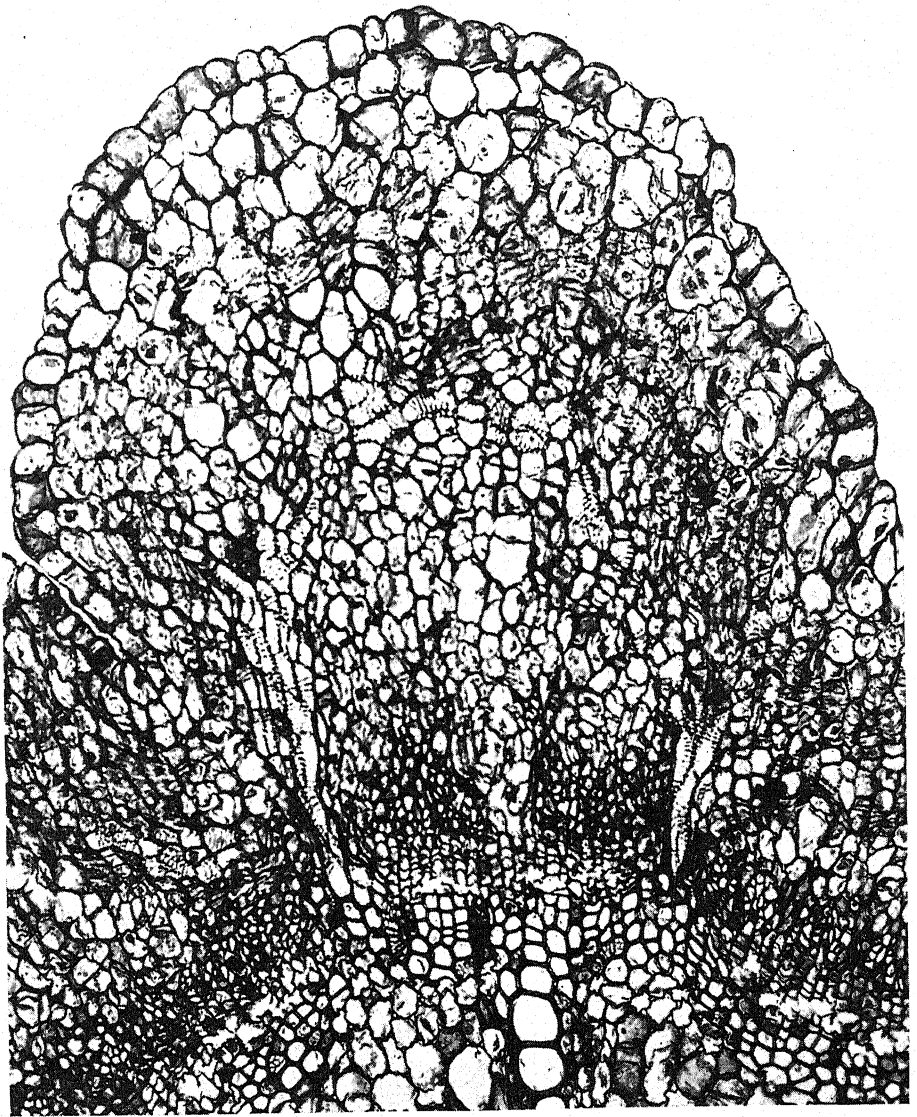


FIG. 9.—Section 86 hours after treatment. Many endodermal derivatives have matured as tracheids, especially above the rays. Most of the cortical cells have divided, some derivatives mature as tracheids, others as bands of meristematic cells which may differentiate as vascular elements. In the secondary phloem is a group of meristematic cells derived from activity of the parenchyma. These cells may differentiate and give rise to a vascular strand, or die and disintegrate as in fig. 11. Cambium has continued development, and from it has differentiated secondary tissue much as if the stem had not been decapitated.

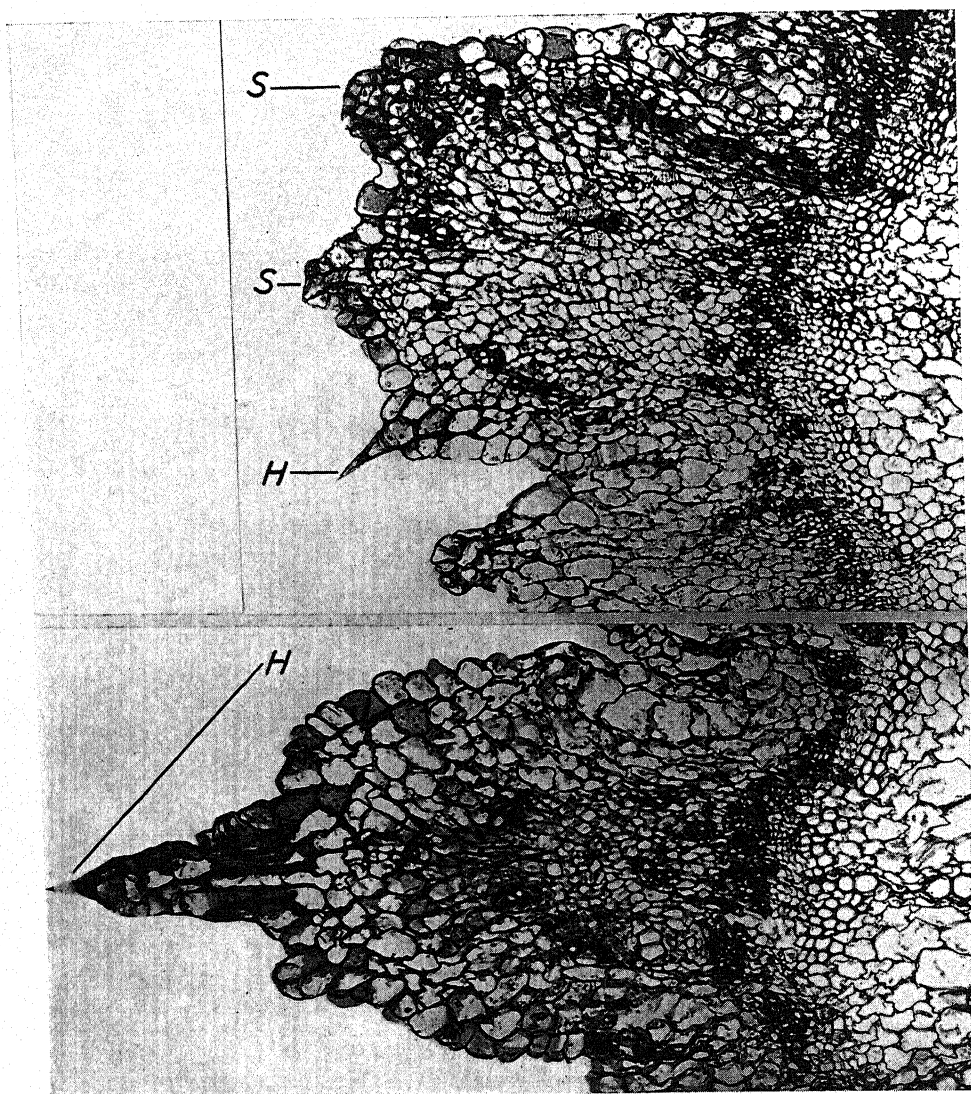


FIG. 10.—Section 186 hours after treatment. *A*, characteristic tracheids derived from endodermal and other cortical cells, and bands of vascular and provascular tissue which extend to cones of tissue associated with development of cells beneath a stoma. *B*, some of these same structures are obvious, and an epidermal hair has been pushed outward by development of a narrow cone of tissue derived from cortical parenchyma. In similar instances, which are common, the epidermal cells divide radially and enlarge so that the epidermis remains continuous and unbroken.



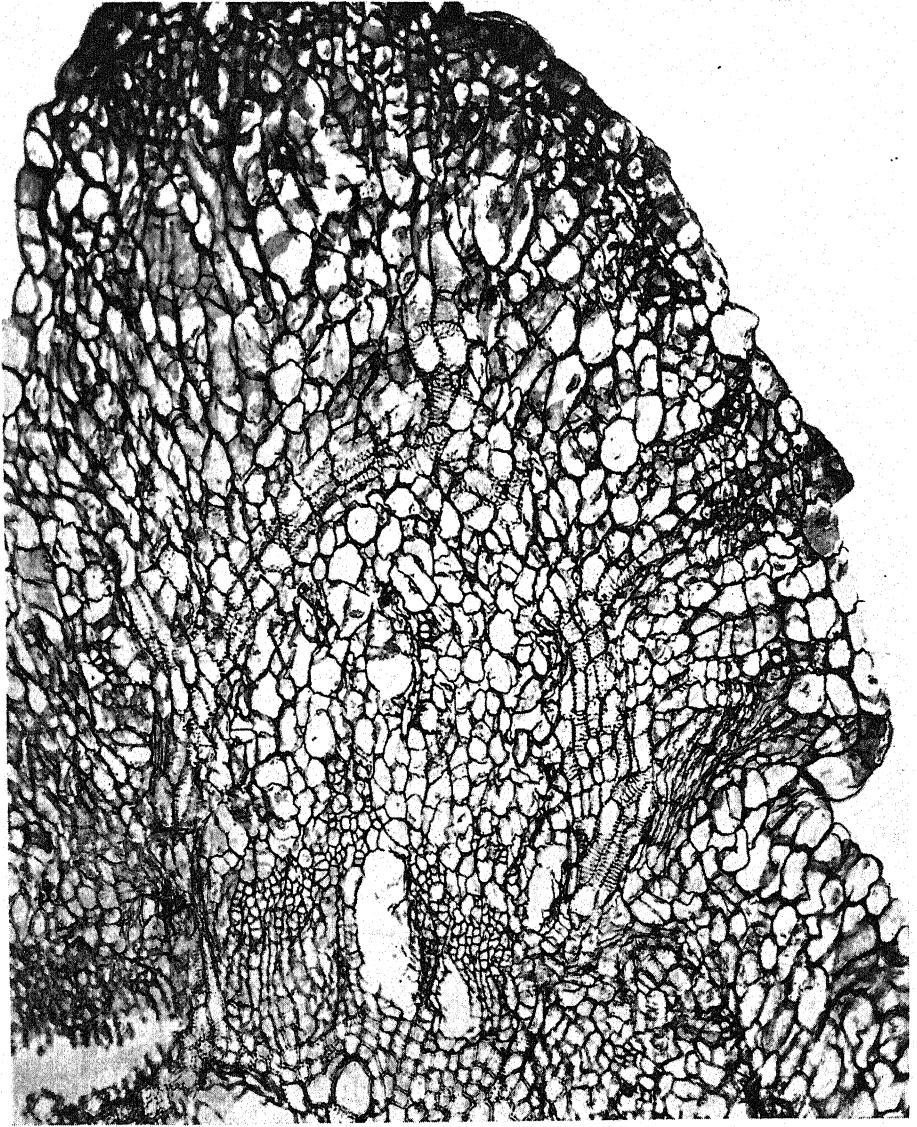


FIG. 11.—Section near treated surface 180 hours after application of pollen extract-lanolin mixture. Although many cells are still actively proliferating, particularly just beneath the epidermis, many also have matured and are no longer actively dividing. In some instances the cells have died and cracks are being formed as result of disintegration and breaking. Many tumors develop only slightly beyond this stage, although some continue to grow or at least remain alive for much longer periods.

The same type of development continues for considerable time, but 180–200 hours after treatment the apical tumors enlarge very slowly or not at all. They may remain alive for much longer periods but many of them began to die and shrivel at about that time. At 180 hours the cells of cortical parenchyma may still be active, especially just beneath the epidermis, where there are groups of meristematic cells separated by others which have enlarged and matured as thin-walled cells or as tracheids. The groups of cells are generally connected with strands of similar active cells extending to the tracheids matured from endodermal derivatives, or they may be in direct connection with the vascular elements of the stele through derivatives of the rays. As noted, these strands frequently terminate beneath a stoma, and the guard cells are pushed outward by the development and maturation of cortical tissues beneath them, until they stand at the apices of conical or variously shaped masses of tissues of considerable size, the epidermis remaining continuous owing to the continued division and enlargement of its cells. In somewhat similar manner the epidermal hairs are frequently carried outward at the ends of long points or cones of tissue, but in these cases it is uncommon for a vascular strand to be differentiated to the surface as it is below the guard cells (fig. 10). The cells of the phloem may or may not show proliferation; if at all, it is only slight; but they remain turgid and show no sign of collapse or disintegration. In a few instances scattered tracheids surrounded by sieve tubes and companion cells are differentiated in the primary phloem. Frequently rifts and cracks occur indiscriminately among the various tissues which constitute the tumor, despite the fact that actual meristematic regions are also present (fig. 11).

Compared with control plants treated with lanolin only, those to which the pollen extract has been applied show continued development of the pith, rays, cambium, secondary xylem, and phloem, which closely parallels undecapitated and untreated internodes of the same age; whereas the controls generally cease to grow, the tissues collapsing and shriveling. Occasionally such a control may continue some development, usually a proliferation of the phloem cells (fig. 12), as has already been described (9). In other cases, the cells of the endodermis, the outer portion of the rays (the cortical parenchyma particularly), and those of the epidermis enlarge greatly, become actively meristematic, and derivatives from all but the epidermis differentiate as parenchymatous tissue and vascular elements, especially as tracheids. Many of these are elongate, long pointed, and coarsely reticulate.

In general, histological responses following the application of pollen extract-lanolin mixtures to the cut surface of decapitated internodes of bean stems were unlike those recorded for indoleacetic and naphthaleneacetic acids (8), but more closely resemble those that resulted following the application of tryptophane (11).

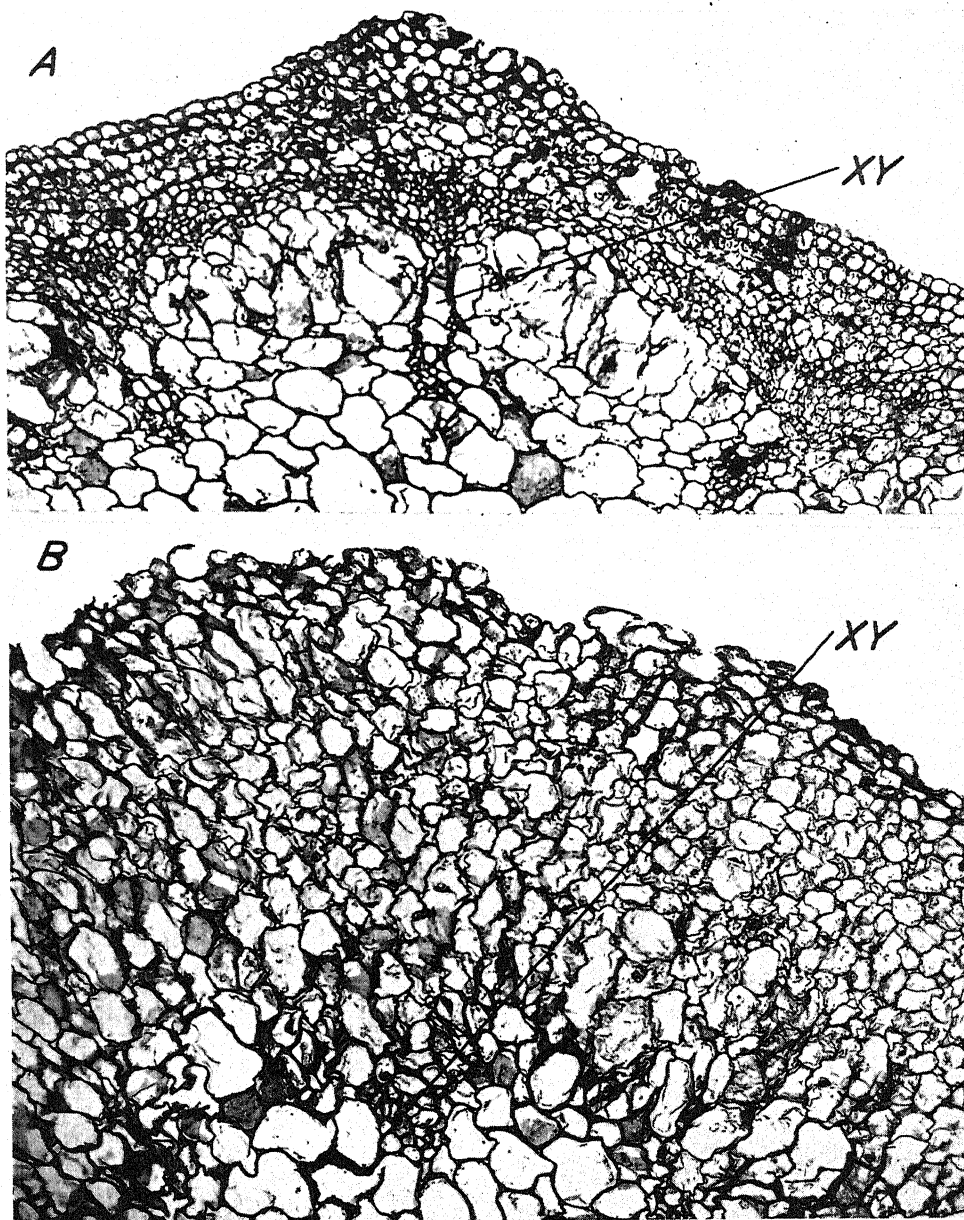


FIG. 12.—*A*, decapitated stem 86 hours after treatment with lanolin only. Although some cells were still alive there was little development after decapitation, and many cells have disintegrated. *B*, similar section immediately below treated surface 180 hours after decapitation and treatment. Phloem parenchyma proliferated to some extent. Most cells have died, although tumors of this character may continue development for considerable time.



### Summary

1. Extracts from pollen of *Zea mays* mixed with lanolin were applied to certain vegetative parts of various species of plants, particularly to those of *Phaseolus*.

2. Application of the extract in the form of a ring around the stems of bean plants resulted in marked internodal elongation, which was associated mainly with an increase in the length of the cells of the various tissues, rather than with an increase in the rate of cell division. Small amounts of indoleacetic acid applied in a similar way also resulted in increased linear growth of internodes, but to a less extent.

3. Greatest increase in linear growth of bean stems resulted when the extract was applied to internodes of plants grown in light of an intensity and quality most favorable for the development of short sturdy stems, and there was little or no difference between internodal elongation of treated and control plants grown under light conditions that favored etiolation.

4. Small tumors generally resulted following application of the pollen extract to the cut surface of decapitated second internodes of bean plants. Histological responses associated with the growth of these tumors were unlike those that resulted following the application of relatively concentrated mixtures of indoleacetic or naphthaleneacetic acids and lanolin, but more closely resemble those recorded for tryptophane.

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# INFLUENCE OF LOCALIZED LOW TEMPERATURE ON BILOXI SOYBEAN DURING PHOTOPERIODIC INDUCTION

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(WITH TWO FIGURES)

## Introduction

Numerous studies dealing with the effect of the interaction of photoperiod and temperature upon flowering have recently been made (3, 4, 5, 6, 7, 8, 9). In some of these studies the experimental treatments were continued for several weeks whereas in others they were restricted to durations of only a few days. In general, the response to photoperiod has been markedly influenced by variations in temperature.

In Biloxi soybean plants low temperature during photoperiodic induction resulted in a decrease in floral initiation (7). In this study, and in most others of similar nature, the entire plant was subjected to the various treatments imposed, and so it was not clear just what particular process or processes were affected.

Low temperature has been shown to produce a change in the metabolism of the leaf cells (7), and since the leaf is known to be the seat of photoperiodic stimulation in the plant, such changes could account for the different flowering responses observed. It is also possible that low temperature may influence the transfer of materials from the leaves to the growing points. Such effects have been demonstrated in other plants (2) and might be expected in the soybean. More or less complete inhibition of the transfer of food materials or of any special flower-inducing substance that may be formed in the leaves would probably result in a difference in the extent of flower-bud initiation induced by a given treatment. Reduction in flower-bud formation could also result from the action of low temperature upon the meristems themselves, although these growing points may be supplied with adequate amounts of food and growth-regulating substances.

The data presented here deal particularly with the influence of low temperature applied to the growing points of stems and to the petioles of leaves during the period of photoperiodic induction.

## Material and methods

In preliminary attempts to chill the petioles of leaves several methods were tried before a satisfactory one was developed. In the method finally used, cold

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water was circulated through a glass cylinder (fig. 1) sealed about the petiole. It was in actual contact with the petiole and produced a uniform temperature throughout the tissue inclosed in the cell.

The cooling cells were made of glass tubing approximately  $1\frac{1}{2}$  inches in diameter and 2 inches in length. The cells were fitted with rubber stoppers on which shoulders were cut in such a way that they could be inserted for  $\frac{1}{4}$  inch into each end of the cell, thereby providing a uniformly effective cooling length of  $1\frac{1}{2}$  inches.

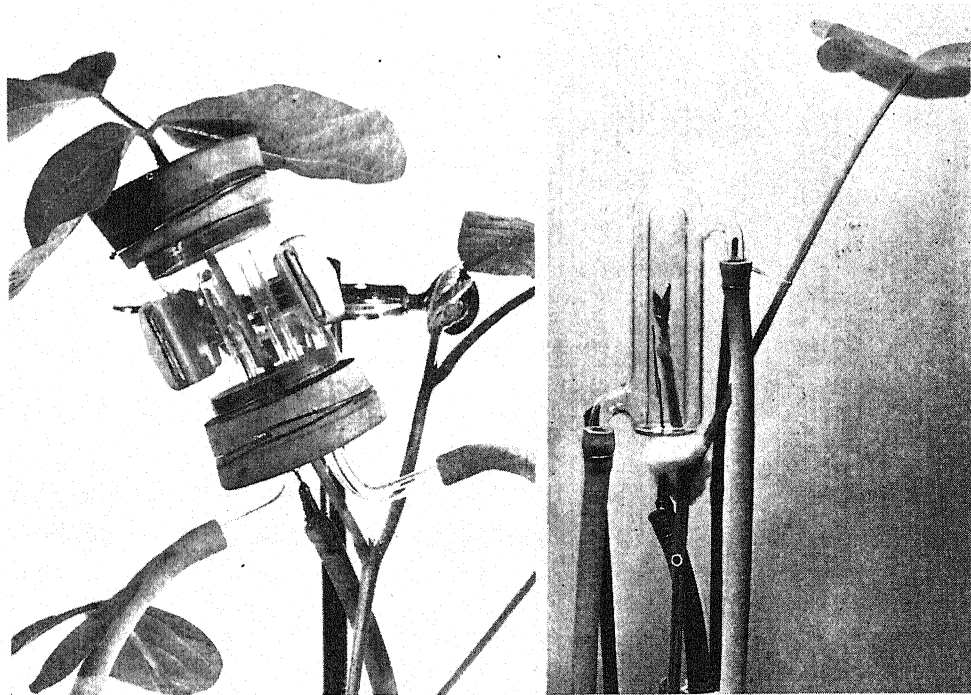


FIG. 1.—Apparatus for localized cooling. Petiole cooler on left and tip cooler on right

The stoppers were split to a hole in the center so that they could be placed about the petiole. The lower stopper was fitted with inlet and outlet tubes for circulation of the water and with a well for a thermocouple.

The cooling cells were placed on the petiole by loosely rolling the three leaflets of a leaf and passing them through the glass cell. The stoppers were spread open and grafting wax of a low melting point was placed in the hole that received the petiole. When the petioles were in place the stoppers were gradually pressed into position. The stoppers were bound together by means of wire, thus insuring an effective seal between the petiole and the stopper.

Double-walled glass cells were devised to cool the stem tips. These cells (fig. 1)

consisted of two glass tubes sealed together in such a way that the stem tip with its terminal bud could be inserted from the base. Inlet and outlet tubes were sealed to the sides. The effective thickness of the layer between the two tubes was approximately 5 mm., and the effective length of the cells was 8.5 cm. The stem tip and a thermocouple wire were inserted into the cells when the apparatus was set up, and a cotton plug was placed in the bottom. In figure 1 this plug has been partly withdrawn to show the lower part of the cell.

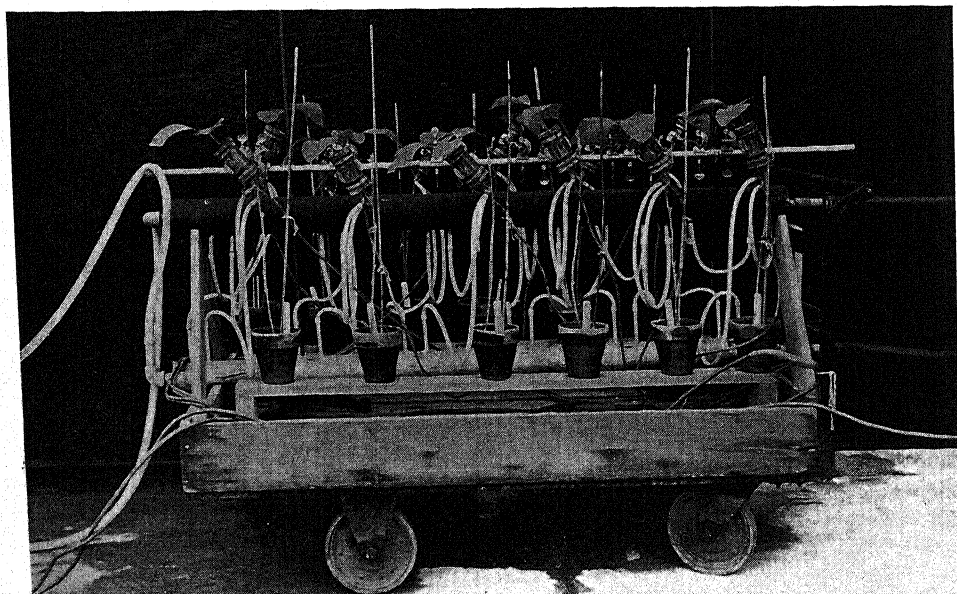


FIG. 2.—General view of plants and apparatus for typical petiole-cooling experiment. A similar truck was used for tip-cooling cells.

An insulated tank containing 30 gallons of water and a refrigerator to cool it were installed on a warehouse truck. The water was circulated to the petiole and tip-cooling cells through manifolds mounted on two other trucks (fig. 2).

Temperatures of each cell and of the water in the tank were recorded three or more times daily. Temperatures of the cells were obtained from thermocouples read with a portable Leeds and Northrup potentiometer; those of the tank were taken with a mercury thermometer. The temperatures reported for the individual experiments varied  $\pm 2^{\circ}$  C. for short periods during the day, but were very close to the temperatures indicated during the night.

The various lots of plants were grown in the greenhouse during the months of November, 1940, to April, 1941, inclusive. They received the natural photoperiods then prevailing and Mazda light from sundown until midnight. The plants varied in age from 42 to 65 days at the beginning of the treatments.

In the experiments involving cooling of the stem tips, all large leaves were removed from experimental and control plants except the one youngest fully-expanded leaf on each plant. The two partially-expanded leaves immediately above were also removed in all experiments, and in a few cases three such leaves were removed. All buds were removed from the plants except those in the axils of the unexpanded leaves remaining at the tip of the stem. These buds were within the cooling cells and were therefore subjected to the temperature maintained during any particular experiment. The only part of the plant capable of flower-bud initiation was therefore restricted to the region where the temperature was controlled.

Defoliation for the petiole-cooling experiments was done as described for the tip-cooling experiments, except that in some experiments the two most recently fully-expanded leaves were left on the plant. No buds were removed from plants of the petiole-cooling experiments.

Induction treatments consisting of four 8-hour photoperiods were used in all the experiments. In those involving plants with single leaves the short days were obtained by placing the group of three trucks in a dark chamber at 4:00 P.M. and removing them at 8:00 A.M. In those experiments involving plants with two leaves, one on short and one on long photoperiod, the short photoperiod was provided by means of black cloth bags as described previously (1). Long day was provided by extending the natural photoperiod with Mazda light. In these experiments the trucks were left in the greenhouse. The control plants were defoliated and disbudded in exactly the same way as the experimental plants with which they were associated.

After the treatments were completed, the plants were returned to long photoperiods and dissected 2-3 weeks after the start of the induction treatment.

### Experimental results

Petiole-cooling experiments were begun in November, 1940, and continued through April, 1941. During this time a wide range of environmental conditions, such as variability in light intensity and greenhouse temperature, was encountered. The age and general condition of the plants also varied from one experiment to another. In all the experiments, however, it was possible to demonstrate a retarding influence on floral initiation when low temperature was applied (table 1).

The first series of tests dealt with plants which had been defoliated to one fully-expanded trifoliate leaf and included experiments conducted at various temperatures. These single-leaved plants received an induction treatment consisting of four 8-hour photoperiods. When the temperature of the petiole was maintained at 3° C. during the induction period, the production of flower primordia was greatly inhibited. In three experiments run during November, only one plant of ten in each lot formed flower primordia. In the other experiment, in which the petiole was maintained at 3° C., eight of ten plants formed primordia but the total number of

flower buds was only fourteen, while similar control plants produced thirty-seven. When the temperature of the petiole was raised to 6° C., eight of eleven plants produced flower primordia. At this temperature the cooled lot produced a total of twenty-five flower buds while the control plants produced forty-six.

When water at 25° C. was circulated around the petioles, all the plants formed flower primordia. The total number formed on the ten plants was twenty-six.

TABLE 1  
FLORAL INITIATION AS AFFECTED BY COOLING PETIOLES OF  
BILOXI SOYBEAN DURING PHOTOPERIODIC INDUCTION

TREATMENT STARTED	No. OF NODE WHERE LEAF RE- MAINED	TEM- PERA- TURE OF WA- TER (° C.)	PLANTS WITH COOLING CELLS ON PETIOLES					PLANTS WITHOUT COOLING CELLS ON PETIOLES				
			PLANTS PER LOT	PLANTS WITH FL. PR.	FL. PR. PER LOT	TOTAL NO. OF NODES	IN- CREASE IN LENGTH OF LEAF* (CM.)	PLANTS PER LOT	PLANTS WITH FL. PR.	FL. PR. PER LOT	TOTAL NO. OF NODES	IN- CREASE IN LENGTH OF LEAF* (CM.)
11/8/40.....	5	3	10	1	2	17.9	0.32	10	6	8	18.7	1.79
11/12/40.....	6	3	10	1	1	19.2	0.40	10	10	23	19.9	2.13
11/22/40.....	6	3	10	1	2	18.8	0.49	10	8	17	19.4	0.98
2/6/41.....	6	3	10	8	14	21.3	0.07	10	10	37	22.4	0.58
2/18/41.....	6	6	11	8	25	22.8	1.04	12	12	46	23.7	1.57
4/28/41.....	5	7	12	7	15	19.8	2.37	12	12	47	20.7	2.84
11/18/40.....	7	25	10	10	26	20.9	1.27	10	10	32	21.0	0.88
11/26/40.....	4, 5	3	10	1	1	19.5	0.96	10	10	29	19.9	1.12
3/17/41.....	5, 6	3	12	2	2	21.2	1.30	12	11	21	21.7	1.36
12/2/40.....	5, 6	3	10	1	4	21.1	1.34	10	10	23	21.1	0.99
2/25/41.....	6, 7	3	12	4	10	22.0	0.70	12	11	32	22.4	0.72
4/21/41.....	8, 9	5	12	7	15	26.2	0.88	12	12	47	26.6	0.86
3/24/41.....	5, 6	6	12	8	15	22.2	4.99	12	12	32	22.8	4.69
3/3/41.....	6, 7	6	12	9	20	24.6	1.42	12	12	43	23.5	0.93
4/14/41.....	7, 8	10	12	12	55	25.0	4.16	12	12	64	25.2	3.29

\* Center leaflet of leaf at base of terminal bud measured.

Similar plants receiving the same photoinductive treatment but not having cooled petioles produced thirty-two flower primordia on ten plants. This experiment indicates that the mere presence of water around the petiole does not interfere with movement of the flower-forming stimulus from the leaf to growing points.

In experiments at 3° C. the increase in length of the center leaflet of the lowermost leaf in the terminal bud was much greater in the controls than in the plants with the cooled petioles. The total number of nodes in the main axis of the control plants was also greater in these experiments than in the plants with cooled petioles. These facts indicate that growth during the photoinductive treatment was greatly reduced.

Since all the food for the growth of these plants was furnished by a single leaf, the petiole of which was cooled, the reduced growth relative to that of the controls was to be expected. It has been shown (2) that this procedure greatly reduces the translocation of carbohydrates. Since apparently very few structures were initiated during the induction period of these experiments, it could not definitely be shown that a flower-forming stimulus was not present in the growing points. To determine whether or not such a stimulus had passed through the cooled petiole, it was necessary to insure the production of new structures upon which the stimulus might act. This was provided by leaving on the plant a second leaf which was not cooled and which was kept on long photoperiod. This leaf supplied food to the growing point, but—on the basis of previous experiments—did not influence the capacity of the remainder of the plant to form flower primordia. The uppermost leaf was given short photoperiods and its petiole was cooled to various temperatures as in the previous experiments. The data with two-leaved plants in table 1 show that growth, as measured by increase in leaf length and total number of nodes on the main axis, was approximately the same whether or not the petioles of the uppermost leaf were cooled. This indicates that growth of the terminals was maintained at a rate comparable with that of the controls. In all the experiments at this temperature the total number of flower primordia produced on the plants with cooled petioles was much less than on the controls, indicating that the movement of the flower-forming stimulus was definitely reduced when the petioles were cooled to 3° C.

When the temperature was increased to 5° or 6° C., more of the plants with cooled petioles produced flower buds. At 10° C. all the plants in the cooled lot produced flower buds, and the total number approached the total number produced by the controls.

In the tip-cooling experiments low temperature applied to the stem terminals resulted in marked reduction in flower-bud initiation (table 2). These plants were disbudded at all nodes, except those in the terminal buds, which were surrounded by the cooling cells. All the leaves, except the one most recently fully expanded and the small unexpanded leaves in the terminal bud, were removed. Control plants were disbudded and defoliated in the same manner.

When the terminal bud was cooled to 3° C. no flower primordia were formed on two lots of eleven plants each. All the twenty-two control plants of these two lots produced flower primordia. One lot formed twenty-five and the other sixty-three. In another lot cooled to the same temperature seven of the eleven plants formed a total of twelve flower buds. The eleven control plants all initiated flower primordia and formed a total of forty buds.

At a temperature of 6° C., twenty-one of the twenty-two plants in two experiments produced a total of forty-one flower buds. All the twenty-two control



plants of these two lots formed flower primordia. The total number formed, however, was more than twice as many as on the tip-cooled plants.

At 10° C. all plants in both the tip-cooled and the control lots initiated flower buds. The number formed by the controls was somewhat less than twice the number formed by the tip-cooled plants. In general, smaller differences in the extent of flower-bud initiation between tip-cooled and control lots resulted as temperature increased.

Increase in length of the leaflets measured was very slight at all temperatures. The increase in the controls, although variable from one experiment to another,

TABLE 2  
FLORAL INITIATION AS AFFECTED BY COOLING STEM TIPS OF  
BILOXI SOYBEAN DURING PHOTOPERIODIC INDUCTION

TREATMENT STARTED	TEMPERATURE OF WATER (° C.)	PLANTS WITH TERMINAL COOLERS					PLANTS WITHOUT TERMINAL COOLERS				
		PLANTS PER LOT	PLANTS WITH FL. PR.	FL. PR. PER LOT	TOTAL NO. OF NODES	IN- CREASE IN LENGTH OF LEAF*	PLANTS PER LOT	PLANTS WITH FL. PR.	FL. PR. PER LOT	TOTAL NO. OF NODES	IN- CREASE IN LENGTH OF LEAF* (CM.)
2/25/41.....	3	11	7	12	22.0	0.02	11	11	40	22.5	0.90
3/17/41.....	3	11	0	.....	20.4	.05	11	11	25	21.9	2.07
4/21/41.....	4	11	0	.....	25.4	.07	11	11	63	27.0	1.07
2/18/41.....	6	11	11	25	22.3	.04	11	11	51	23.8	1.22
3/3/41.....	6	11	10	16	22.2	.03	11	11	39	23.4	1.28
4/14/41.....	10	11	11	32	22.1	0.21	11	11	57	24.6	6.96

\* Center leaflet of leaf at base of terminal bud measured.

was much greater than that of the corresponding tip-cooled lots. Less striking differences but of the same trend occurred in the total number of nodes formed. The temperatures reported in these experiments all appear—on the basis of the type of measurements made—to have inhibited growth to about the same degree. Flower-bud formation, however, was much more markedly suppressed at the lower than at the higher temperatures.

### Discussion

It is known that the leaves of plants are the organs of photoperiodic perception, and that the formation of flower buds resulting from photoperiodic stimulation occurs in any growing points of the stem. This necessitates the transport of the stimulus from the leaves to the growing points. The influence of temperature on the production of flower buds, therefore, could be exerted in at least three ways:

(1) upon the organs of perception (leaves); (2) upon the structures and processes involved in translocation of the stimulus from these organs to the growing points; or (3) upon the growing points themselves. Any one or any combination of these could affect the differentiation of flower buds.

In the case of the petiole-cooling experiments, the effects were limited to the structures and processes of translocation, whereas in the case of the tip-cooling experiments the apical meristems were primarily subjected to the influence of low temperature, although the structures and functions concerned with translocation were also influenced to the extent that the stem was inclosed in the tip cooler. In either case the organs of perception were uninfluenced by the conditions of low temperature, since they were subjected only to the conditions prevailing in the greenhouse. The influence of temperature upon the processes in the leaf, in so far as they pertain to flower-bud formation, are being subjected to further investigation.

When the petioles were cooled to 3° C., floral initiation was greatly suppressed or completely inhibited. At 10° C., however, most of the plants formed flower buds, although less extensively than the controls. In the tip-cooling experiments it was also found that flower-bud formation could be greatly limited by a very low temperature, but at 10° C. floral initiation was fairly abundant on all the plants. Thus only when the processes involved in translocation and in formation of new structures were subjected to extremely low temperatures was flower-bud formation markedly suppressed.

In previous experiments (7) with soybean, in which whole plants were subjected to low temperature, it was found that at 55° F.—12.8° C. no flower primordia were formed. On the basis of the present experiments, their failure to form at that temperature was not a result of limitation of the transport of the flower-forming stimulus nor of the production of new structures at the growing points.

### Summary

1. The influence of low temperature in suppressing flower-bud initiation in Biloxi soybean has been considered from three points of view: its influence upon the differentiation of flower buds from the meristems; its influence on translocation of a flower-forming stimulus from the leaves to the growing points; and its influence upon those processes in the leaf that bring about flower-bud formation. Data are presented on the first two of these points; the last is being subjected to further investigation.

2. Fewer flower buds were formed in response to a 4-day induction treatment on plants bearing a single leaf if the terminal buds or the petioles were cooled to 3° C. than if they were not cooled. When the temperature was raised to 10° C. the inhibiting effect on flowering decreased in both types of experiments, but even at

this temperature only slight growth of the terminals occurred during the induction treatment.

3. Petiole-cooling experiments involving plants with two leaves indicate that inhibition of flowering resulted from the influence of low temperature on the transport of a flower-forming stimulus.

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## EFFECTIVENESS OF TRYPTOPHANE MIXTURES AS GROWTH REGULATORS

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(WITH TWO FIGURES)

It has been shown (1) that a lanolin mixture containing 2 per cent by weight of l-tryptophane is capable of inducing gall formation when applied to the cut surface of decapitated bean seedlings. In an attempt to extend the experiments reported, another lot of tryptophane-lanolin mixture was tried. It was observed that gall formation took place much more slowly, but that when the original mixture made up in 1939 was again tried, the plants responded to it in the same way as they had done previously. About 10 cu. mm. of each of the two mixtures was then applied unilaterally, 15 mm. below the tip of *Avena* coleoptiles which were grown and decapitated twice as they are for the *Avena* test (4). The result was that the original 1939 mixture induced a very great bending response whereas the second lot caused no bending whatsoever (fig. 1).

It was then decided to try a more extensive series of tests using l-tryptophane from several sources, made up both as mixtures with lanolin and in aqueous solution mixed with agar. Five lots were used as follows: the original sample (1) furnished by the Eastman Kodak Company in 1939; two other samples (2, 3) from the same company, one secured in 1940 and the other in 1941; a fourth sample (4) furnished by the Pfanstiehl Company in 1941; and a fifth (5) secured from Merck and Company in 1941.

When the last four samples were stirred directly with lanolin and these mixtures applied to *Avena* coleoptiles, there was no subsequent curvature. It was observed, however, that the crystals of tryptophane were not very evenly dispersed throughout the lanolin. To secure a more uniform mixture, another method of preparing the mixtures was used. To 100 mg. of each of the samples of tryptophane, 0.2 cc. of water was added, 5 gm. of lanolin was melted and this quantity poured into each of the respective vials containing the tryptophane. These were then heated in a water bath at 100° C. and stirred vigorously. After several minutes the vials were removed from the water bath and the stirring continued until the mixture solidified. Microscopic examination showed that there was a much more nearly uniform dispersion of the tryptophane in the lanolin and the crystals were much smaller (fig. 2). When these mixtures were applied to decapitated bean plants, tumors were formed to the same degree and in the same length of time in all instances as with the East-

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man no. 1 sample. One-gm. aliquots of each of these samples of tryptophane were dissolved in 250 cc. of water and autoclaved for 20 minutes at 120-lb. pressure. The water was then evaporated off on a steam bath. The residue was dried overnight at 80° C. Each of the residues was made up as a 2 per cent lanolin mixture as just described, and in water solution. When the lanolin mixtures of these autoclaved samples were tested on beans, the results were similar to those obtained with the lanolin mixtures last described.

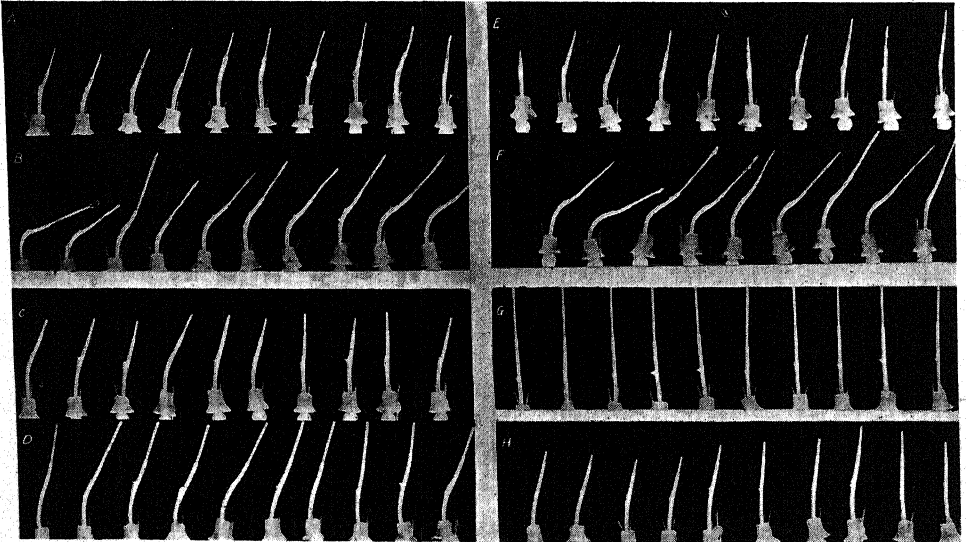


FIG. 1.—Response of *Avena* coleoptiles to unilateral applications of 2% mixtures of tryptophane in lanolin: A, Eastman 1939, original mixture, 1½ hours after application; B, same after 20 hours; C, Eastman 1939, autoclaved, 1½ hours; D, same after 20 hours; E, Eastman 1941, very finely divided and well dispersed mixture, 1½ hours; F, same after 20 hours; G, Eastman 1939, large crystals not well dispersed, 20 hours; H, Pfanstiehl 1941, 1½ hours, sample prepared by adding 0.2 cc. water and melted lanolin, then stirring vigorously until cooled.

A tryptophane-lanolin mixture still more finely divided and uniformly dispersed was obtained by dissolving 100 mg. of tryptophane, lot 3, in about 5 cc. of hot water. This solution was then added to 5 gm. of lanolin heated in an oil bath to 120–140° C. The water was evaporated off under reduced pressure. Microscopic examination showed no large distinguishable crystals (fig. 2). Application of this mixture to bean plants resulted in the formation of tumors somewhat more rapidly than any of the other mixtures previously tried.

When the original tryptophane-lanolin mixture prepared in 1939 was applied as a narrow band around the first internodes of young bean seedlings, as had been done by MITCHELL and WHITEHEAD (2) with a pollen extract-lanolin mixture,

there was elongation of such internodes to a greater degree than in control plants. One week following treatment, when elongation had practically ceased, those internodes to which tryptophane had been applied were significantly longer than the controls. All the plants treated with the tryptophane-lanolin mixtures made up

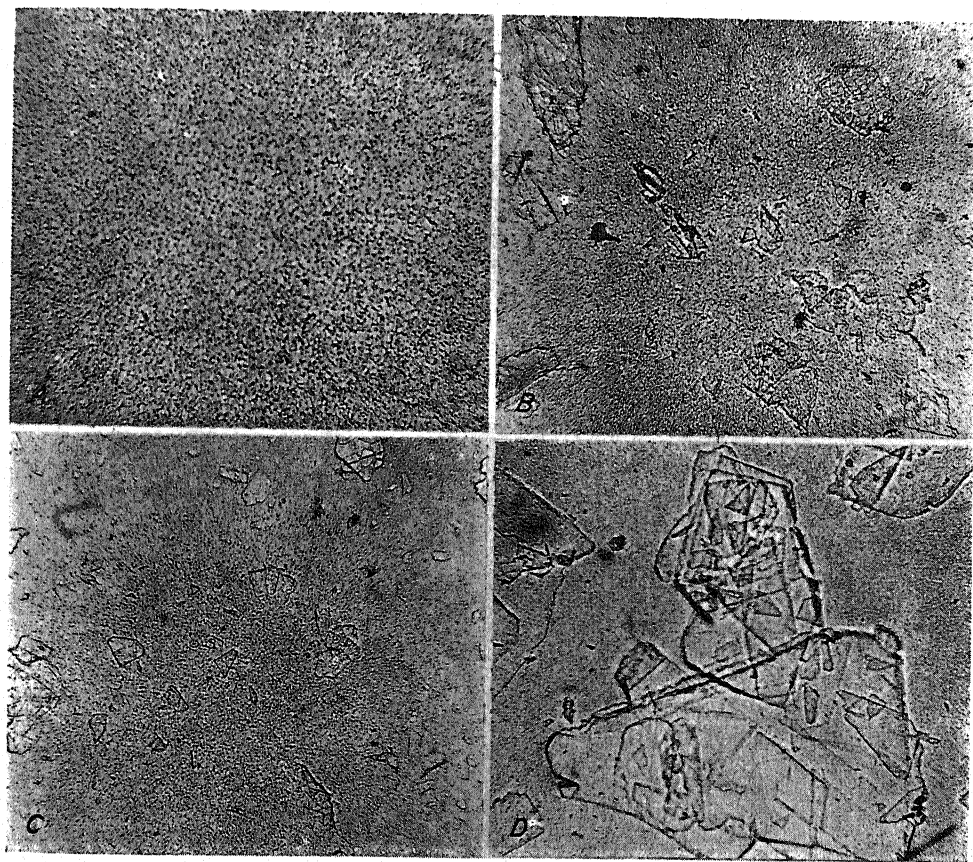


FIG. 2.—Two per cent mixtures of tryptophane in lanolin: A, same as E in fig. 1; B, Eastman 1941 prepared as H in fig. 1; C, original mixture, Eastman 1939; D, same as G in fig. 1. All  $\times 100$ .

by adding 0.2 cc. of water to the tryptophane before mixing were approximately 45 per cent longer than controls. Application of the mixture in which the tryptophane was finely divided resulted in an 80 per cent increase in length of the first internode. In this particular experiment elongation following application of corn pollen extract-lanolin mixture was 98 per cent.

The standard *Avena* test showed that an aqueous solution of Eastman 1939 tryptophane in agar caused the greatest amount of growth promotion. Pfanstiehl tryptophane 1941, lot 5, caused practically none (table 1). Results similar to those

recorded in the table were obtained with tryptophane autoclaved as described. Thus heating tryptophane, as in preparing the lanolin mixtures, apparently does not increase its growth-promoting properties.

All the lanolin mixtures of tryptophane containing 0.2 cc. of water, including the Pfanstiehl mixture, resulted in growth promotion when applied as described to *Avena* coleoptiles. The increased growth was apparent  $1\frac{1}{2}$  hours after application (fig. 1; table 1).

This discrepancy in the action of the Pfanstiehl tryptophane—when applied in lanolin to the side of a coleoptile and when applied as in the *Avena* test at the top of the coleoptile in a water solution in agar—may be due to several differences in

TABLE 1  
RESPONSE OF AVENA COLEOPTILES TO TRYPTOPHANE  $1\frac{1}{2}$  AND 20 HOURS  
AFTER APPLICATION. AS DEGREES CURVATURE PER TWELVE  
COLEOPTILES WITH STANDARD ERROR

SOURCE	STANDARD AVENA TEST 50 MG./L. $1\frac{1}{2}$ HOURS	UNILATERAL APPLICATION OF 2% LANOLIN MIXTURE	
		$1\frac{1}{2}$ HOURS	20 HOURS
Eastman 1939.....	$10.5 \pm 0.7$	$11.7 \pm 1.6$	$17.6 \pm 1.6$
Eastman 1940.....	$6.5 \pm 1.3$	$9.3 \pm 0.9$	$19.9 \pm 1.7$
Eastman 1941.....	$6.0 \pm 0.3$	$5.5 \pm 1.5$	$15.2 \pm 2.6$
Merck 1941.....	$6.5 \pm 1.2$	$6.5 \pm 1.6$	$13.7 \pm 1.7$
Pfanstiehl 1941.....	$1.0 \pm 0.4$	$7.5 \pm 1.5$	$14.6 \pm 1.5$
Eastman 1939, original lanolin mixture.....		$16.8 \pm 1.2$	$53.9 \pm 4.2$
Eastman 1941, finely dispersed lanolin mixture.....		$11.1 \pm 2.1$	$53.1 \pm 3.3$

the methods of application: (a) tryptophane applied in lanolin directly to the region of elongation of the *Avena* coleoptile does not have to travel downward 15 mm. or so to reach the presumed site of action; (b) lanolin itself has very rapid intercellular penetration (3), and thus might carry tryptophane along with it directly to the cells in which it was to act; (c) the area of contact of the lanolin on the side of an *Avena* coleoptile is much greater than that of an agar block on the top cut surface.

Unilateral application to *Avena* coleoptiles of the lanolin-tryptophane mixture, in which that tryptophane was finely divided, resulted in growth-promotion curvatures as great as those from the original 1939 mixture.

It is obvious that in preparing mixtures of lanolin and tryptophane—and probably other synthetic growth-regulating substances as well—considerable care should be taken to assure uniform, finely divided dispersion of the substance in lanolin, and that results may vary greatly, depending upon the character of the

mixture. Whether the tryptophane is changed in the process of preparation, or whether there may be other substances present in the original samples of tryptophane, remains to be determined. If other substances are present in such samples, they apparently occur in all that were used.

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# MEGASPOROGENESIS AND DEVELOPMENT OF THE MEGAGAMETOPHYTE IN NOTHOSCORDUM BIVALVE

MARY LOUISE ECKLES

(WITH NINE FIGURES)

## Introduction

The genus *Nothoscordum*, a member of the Liliaceae, is closely related to *Allium*; in fact, SMALL (6) places it in the Alliaceae. Over twenty species have been reported, only two of which occur in the United States. *Nothoscordum bivalve* (L.) Britton is found growing in sandy soil, from Virginia to Nebraska and southward into Mexico.

Several types of development of the megagametophyte have been reported in the Liliaceae. SCHNARF (5) and MAHESHWARI (4) report the occurrence of the *Scilla*-type, the *Allium*-type, the *Adoxa*-type, the *Fritillaria*-type, and even one case of the *Oenothera*-type—in *Clintonia borealis* as reported by SMITH (7). In addition, the so-called normal type is of frequent occurrence.

It was thought that megasporogenesis and the development of the megagametophyte in *Nothoscordum bivalve* might conform to one of the aberrant types characterizing other members of the family. However, it was found to follow the more typical development.

Few studies have been made of *N. bivalve*. Polyembryony in *N. fragrans* has been reported by several workers, STRASBURGER (8) being one of the earliest. KOERPERICH (3) investigated mitosis in *N. fragrans* and found the somatic chromosome number to be 16. ANDERSON (1) found the haploid number in *N. bivalve* to be 9. BEAL (2) described microsporogenesis and chromosome behavior in *N. bivalve* and confirmed ANDERSON's count. Megagametophyte formation in this species has not been described.

## Material and methods

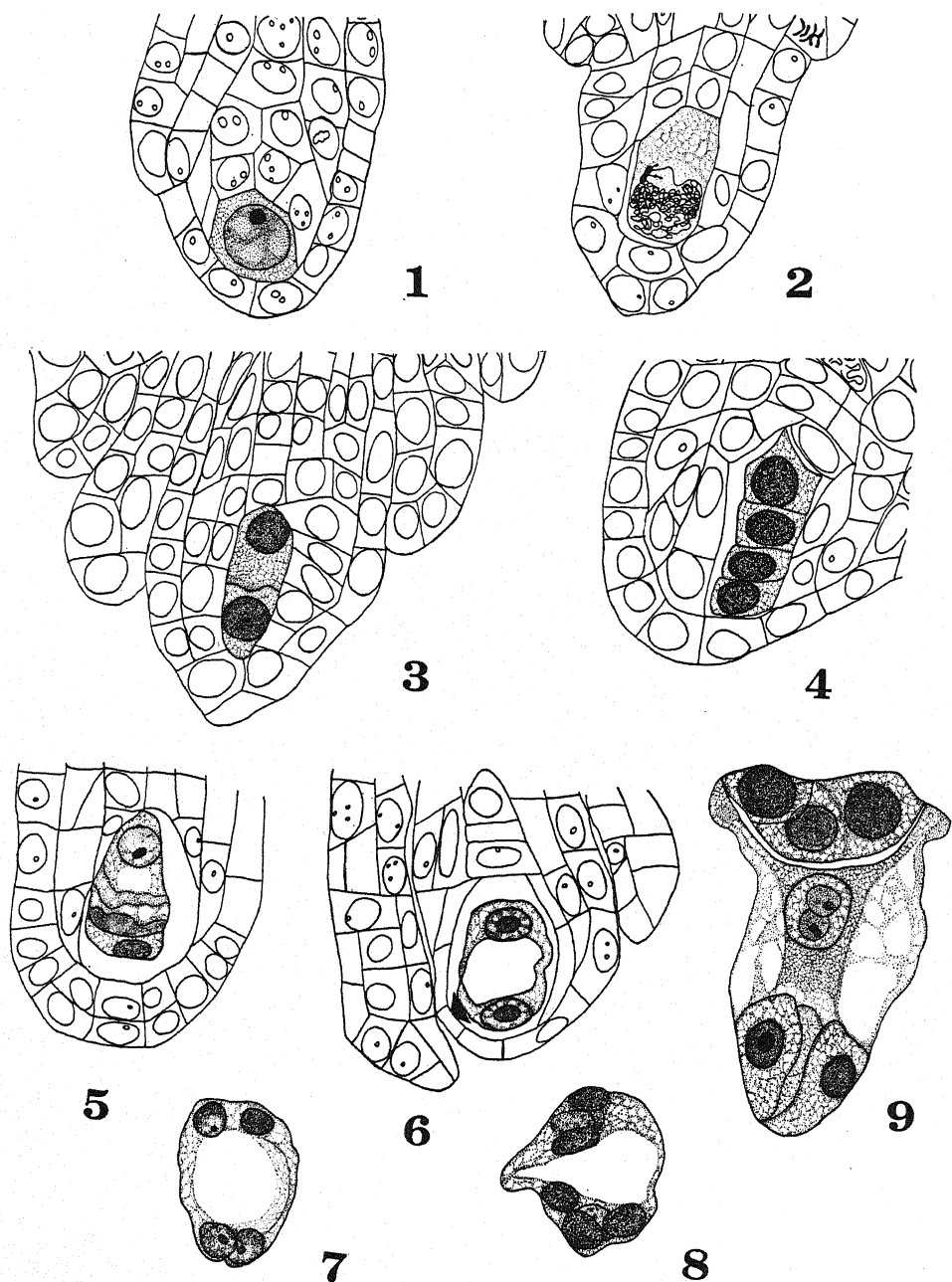
The material used in this study was collected on the campus of Mississippi State College. Flowers of various ages were collected, ranging from buds just emerging from the bulb to flowers that had been open several days. In all the early stages the entire floral structure, with only the two bracts removed, was fixed; in the older flowers only the pistil was used. To insure good penetration of the fixative, the tips of the buds were cut off, as was most of the style in the older flowers. The material was placed in Randolph's modification of Navashin's

fixative, and since it did not sink readily, a water-pressure pump was used. After washing in water, dehydrating in various grades of alcohol, and clearing with chloroform, the material was imbedded in paraffin. Longitudinal sections were cut 10–25  $\mu$  in thickness. Some of these were stained with Flemming's triple stain, some in iron-alum haematoxylin, and some in iron-alum haematoxylin with orange gold in clove oil as a counter-stain. The last-named proved the most satisfactory.

### Observations

The archesporial cell functions as the megaspore mother cell. This is easily distinguished from other nucellar cells by its large size and its hypodermal position. In section, the nucleus fills nearly three-fourths of the cell and contains one to several nucleoli of varying size. The cytoplasm contains numerous small vacuoles but no large ones (fig. 1). The cell elongates somewhat before undergoing the meiotic divisions to form a row of four. The nucleus during synizesis of the first meiotic divisions is shown in figure 2. After the first division the two daughter nuclei are separated by a distinct wall (fig. 3). A second nuclear and cell division form the four megaspores (fig. 4). The one nearest the chalazal end of the ovule is destined to be the functional megaspore, and from the first it is the largest of the four. As the functional megaspore begins to enlarge, the other three disintegrate, the one next to the functional megaspore initiating the disintegration and the others following in succession. As the non-functional spores disappear, the functional one enlarges and its cytoplasm becomes greatly vacuolated (fig. 5). The disappearance of the disintegrating megaspores is not complete until the nucleus of the functional megaspore has divided to form two nuclei, one of which migrates to each end of the cell (fig. 6). The megagametophyte then begins to enlarge at the expense of the surrounding cells, whose remnants appear as darkly staining streaks. In most cases a rather large central vacuole, formed by coalescence of many small vacuoles, appears between the two nuclei, each of which contains a conspicuous nucleolus. Each nucleus divides so that four nuclei, two at each end, are formed (fig. 7). The developing gametophyte continues to enlarge and the four nuclei divide simultaneously. Of the eight nuclei now present (fig. 8), two, one from each end, migrate toward the center of the gametophyte. These polar nuclei meet but do not fuse. Cell delimitation takes place so that seven cells are formed, each with a single nucleus—with the exception of the primary endosperm cell which contains the two polar nuclei.

The mature megagametophyte (fig. 9) thus consists of a central primary endosperm cell containing the two polar nuclei; three antipodal cells at the chalazal end, and an egg and two synergids at the micropylar end. The antipodal cells are closely grouped. Although smaller than the synergids, they contain large nuclei which apparently lack nucleoli. The nuclei in the primary endosperm cell are



FIGS. 1-9.—Figs. 1-4, formation of megaspores: Fig. 1, megaspore mother cell imbedded in nucellus. Fig. 2, same during synizesis. Fig. 3, two cells formed by first division of megaspore mother cell. Fig. 4, row of four megaspores, the chalazal spore the functional one. Figs. 5-9, development of megagametophyte from megaspore: Fig. 5, functional megaspore distinct; other three disintegrating. Fig. 6, 2-nucleate stage following division of the functional megaspore nucleus. Fig. 7, 4-nucleate stage. Fig. 8, early 8-nucleate stage following simultaneous division of nuclei in fig. 7. Fig. 9, mature megagametophyte.

small, each containing one or more nucleoli. The synergids are large and somewhat oval in section, each with a large nucleus containing a nucleolus. The egg lies between the two synergids and projects somewhat into the central region of the megagametophyte. The cytoplasm of the egg is vacuolated and not very dense, and the nucleus, usually containing one nucleolus, does not stain deeply.

About the time that the megaspore mother cell begins to enlarge preparatory to division, the inner integument first appears as a slight bulge (fig. 1). About the time of the first division, the second or outer integument begins to form (fig. 3).

### Summary

1. The archesporial cell serves as the megaspore mother cell.
2. From the megaspore mother cell by two divisions a row of four megaspores is formed.
3. The chalazal megaspore is the functional one; the other three disintegrate, beginning with the one next to the functional megaspore.
4. Development of the megagametophyte follows the typical course; three nuclear divisions result in the formation of eight nuclei, of which two function as polar nuclei, the others becoming the nuclei respectively of the egg, two synergids, and three antipodal cells.

The writer is grateful to Professors J. C. MCKEE and J. F. LOCKE of Mississippi State College and Professor C. E. ALLEN of the University of Wisconsin for their helpful suggestions and criticisms.

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CYTOLOGICAL STUDIES IN RELATION TO THE  
CLASSIFICATION OF THE GENUS  
CALOCHORTUS. II<sup>1</sup>

CONTRIBUTIONS FROM THE HULL BOTANICAL LABORATORY 525

J. M. BEAL

(WITH THREE FIGURES)

In a previous paper (1) the number and morphology of the chromosomes of twenty-eight species and five varieties of *Calochortus* were reported. The taxonomic arrangement followed therein had been furnished by Dr. MARION OWNBEY, who has since published his monograph (2) of the genus, covering a total of fifty-seven species and thirteen varieties. Three additional species have since been investigated and their somatic chromosome numbers determined. They represent two of the three sections into which the genus is divided, and they conform in chromosome numbers with the other species previously investigated cytologically and assigned to these sections.

Bulbs of *C. lobbii* were secured from Mr. Carl Purdy, Ukiah, California, and those of *C. weedii* var. *vestus* and *C. barbatus* were kindly supplied by Mr. William M. James, Santa Barbara, California. *C. weedii* var. *vestus* had been collected locally, while *C. barbatus* had been secured by Mr. James from Mr. George B. Hinton, Mina Rincon via Toluca, Mexico. The bulbs were potted in a sandy loam soil and kept in a cool room in the greenhouse until roots began to emerge. Fixation was made in La Cour's 2 BE solution and the tips were imbedded in paraffin according to the usual schedule. Transverse sections were cut at 20-25 $\mu$  and stained with the gentian violet iodine stain.

The three species reported here are assigned to the sections and subsections in conformity with OWNBEY's monograph (2).

SECTION I. EUCALOCHORTUS

Subsection 2. Eleganti

*C. lobbii* Purdy.—Diploid, 20 chromosomes (fig. 1). The number agrees with and the morphology of the chromosomes conforms in general with those of other species investigated in the section.

<sup>1</sup> This work was aided in part by a grant from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

## SECTION III. CYCLOBOTHTRA

## Subsection 9. Weediani

*C. weedii* var. *vestus* Purdy.—Diploid, 18 chromosomes (fig. 2). This species agrees in number with that previously determined for *C. plummerae* Greene, a member of the same subsection, but the chromosomes appear somewhat thicker, a condition which may perhaps be caused by the fixing solution.

*C. barbatus* (HBK) Painter.—Diploid, 36 chromosomes (fig. 3). This species is apparently tetraploid since at least certain of its chromosomes occur in quadruplicate.



FIGS. 1-3.—Root tip metaphases: fig. 1, *C. lobbii*,  $2n=20$ ; fig. 2, *C. weedii* var. *vestus*,  $2n=18$ ; fig. 3 *C. barbatus*,  $2n=36$ .

The number of species of the Cyclobothra section thus far investigated is three, and all of them show 9 as the basic number of chromosomes. It would be of interest if still other species were to be studied to determine whether this basic number may be constant for the section.

An interesting feature of the cytological studies of *Calochortus* has been the establishment of the close correlation between the number and morphology of the chromosomes on the one hand and the morphologic and organographic characters on the other, both of which were used by OWNBEY as criteria for the separation of the genus into sections and subsections and the assignment of species under them.

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# WATER CONTENT AND OSMOTIC PRESSURE OF SUN AND SHADE LEAVES OF CERTAIN WOODY PRAIRIE PLANTS

FRANK L. MARSH

A recent study of the water content and osmotic pressure of certain herbaceous prairie plants in the vicinity of Lincoln, Nebraska, emphasized the influence of direct sunlight on the development of relatively high osmotic pressures (2). A similar effect was observed in woody prairie plants of the same locality. These previously unpublished data include results of experiments with sun and shade leaves of *Cornus stolonifera*, *Rhus glabra*, *Sambucus canadensis*, and *Vitis vulpina*.

## Material and methods

Leaves in sun and shade were collected from 1:00 to 2:00 P.M. on sunny days and immediately placed on solid carbon dioxide in small screw-top aluminum cans, where they were frozen crisp in a few minutes. The material was thawed the following morning and the sap expressed immediately under a pressure of 10,000 pounds per square inch. The depression of the freezing point was promptly obtained by an ether-evaporation device (2). Osmotic pressures were found by using the corrected tables of HARRIS (1).

Leaves for water-content determination were secured in amounts of 75 gm. in small, screw-top glass jars. This material was a duplicate of that used in obtaining osmotic pressures and was collected at the same time. Samples were dried at 80° C. for at least 48 hours. Calculations were based on green weight.

Material was obtained from plants growing in a shallow ravine where soil moisture was abundant. Only mature, fully expanded leaves were used. Sun leaves were collected on the south side at the periphery near the top of the plant. Under the intense sunlight of this region, these leaves were definitely thicker and smaller than those situated in the shade only a few inches in from the periphery; planimeter measurements of the areas of upper surfaces of sun leaves of *Cornus* gave an average of 2.7 square inches while the area for shade leaves was 6.4.

The shade leaves were collected at a point just beneath the peripheral canopy. A steady south wind blew at the time, so that the atmosphere surrounding the leaves in both situations had the same relative humidity. Psychrometer readings were the same at the periphery as just beneath it, since such readings are always taken with the wet and dry bulbs shaded. However, the actual temperatures of sun leaves *in situ* were 5-7° F. higher than were those of the shade leaves (table 1).

These temperatures were obtained by folding the illuminated or shaded leaves closely over the thermometer bulb, the resultant readings indicating the internal temperatures of the leaves. The external physical factors of these sun and shade leaves were identical except for the direct sunlight upon the former and diffuse sunlight upon the latter, with accompanying temperature differences.

### Results

Sun leaves of the four species studied had 1.1–11.6 per cent lower water content than the shade leaves (table 1). Osmotic pressures of the former were 2.1–5.4 atmospheres higher than those of the latter.

TABLE 1  
OSMOTIC PRESSURE AND WATER CONTENT OF MATURE SUN AND  
SHADE LEAVES IN VICINITY OF LINCOLN, NEBRASKA

PLANT	HEIGHT (INCHES)	LEAF	LIGHT (FOOT CANDLES)	TEMPERATURE (°F.)	WATER CONTENT (%)	OSMOTIC PRESSURE (ATMOS- PHERES)
Cornus stolonifera.....	60	Shade	175	83	72.2	10.5
		Sun	10,000	89	60.6	15.9
Rhus glabra.....	60	Shade	400	84	66.1	10.7
		Sun	10,000	89	65.0	12.8
Sambucus canadensis...	60	Shade	100	82	84.9	8.8
		Sun	10,000	89	75.8	12.3
Vitis vulpina.....	60	Shade	40	82	76.5	6.4
		Sun	10,000	88	71.8	9.2

### Discussion

It has been shown that where soil moisture is abundant there may be no correlation between water content and osmotic pressure of the tissues of herbaceous prairie plants (2). In such situations, water content may be highest in the same portion of the stem in which there is the greatest amount of solutes. Likewise, in leaves of plants which have abundance of available soil moisture, osmotic pressure may increase at the same time that water content is increasing. As shown in this study, however, sun leaves had higher osmotic pressures and lower water content than shade leaves, regardless of abundance of water at the roots. The higher osmotic pressure of the former was apparently due to the lower water content of the tissues.

In all cases the shade leaves were sufficiently illuminated to produce maximum photosynthesis, but the total amount of infra-red rays was much less than that falling upon the sun leaves. This latter fact is evidenced by the lower temperatures



of the shade leaves. According to SPOEHR (4), about 50 per cent of the total radiant energy of the sun is transformed in the leaf and used in the vaporization of water. It is well known that transpiration in sun leaves is more rapid than in shade leaves. In some plants a linear relation between water loss and light intensity has been determined under a rather wide range of conditions (3). It appears correct to assume that, under the external physical factors of this experiment, photosynthate was as abundant in the shade leaves as in the sun leaves. However, the higher rate of water evaporation from the latter produced a greater concentration of osmotically active substances.

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## METHOD FOR SOFTENING FILBERT BUDS IMBEDDED IN PARAFFIN

C. E. SCHUSTER<sup>1</sup>

Repeated attempts to section filbert buds and filbert pistils have been made by the writer since 1920. Even dissecting out the pistils produced little success, owing to the inability to cut the hairs at the base of the pistils. Since 1935 an intensive study has been carried on, during which time practically all reported methods and materials for such type of work were tried out, as well as many suggested by co-workers. Some improvement over standard methods was made, but not to the degree desirable. The material collected during January-April consistently resisted successful sectioning.

Of the methods and materials tried, soaking the paraffin-imbedded buds in water was best, but even that method failed to give results desired. Too many sections were torn across by the knife, and from some samples no sections were made that could be studied satisfactorily. Blocks set aside in water and forgotten until the blocks were slimy and the water thick with molds and slime gave the clue to part of the methods reported here. No attempt has been made as yet to identify the product or organisms responsible for the action on the imbedded plant material, but trials will be undertaken soon to study and isolate the organisms.

The method as worked out is the result of attempts to section filbert pistils for gross studies of pollen tubes, tube cysts, and similar developments in the pistil. It might not be suited for material intended for study of finer details.

The procedure as finally revised is as follows:

### 1. Solution A

- 10 cc. 2% chromic acid (water solution)
- 100 cc. 95% ethyl alcohol
- 100 cc. 36% glacial acetic acid

### Solution B

- 40 cc. 40% formalin
- 100 cc. 95% ethyl alcohol

Use equal parts of solutions A and B. Unless whole buds are needed for study, dissect out the parts desired, as it is difficult to obtain a good infiltration of paraffin. The material should remain in the killing and fixing agent for at least 24 hours.

<sup>1</sup> Horticulturist, Bureau of Plant Industry, United States Department of Agriculture.

The writer appreciates the assistance of EDNA GEIL and WILLIAM EVENDEN, Laboratory Assistants, in this work. Acknowledgments are made also to Dr. W. B. BOLLEN, Associate Bacteriologist, Oregon Experiment Station, for help and suggestions. Assistance was furnished by the Works Progress Administration under projects W.P. 1656, O.P. 465-94-3-39 and W.P. 2573, O.P. 665-94-3-38.

2. Boil in 50% dioxan for 40 minutes.

Place vials containing buds in a water bath under a ventilating hood. (Dioxan fumes are extremely injurious to health.) After boiling, pour off the dioxan.

3. Use zinc chloride, 10 gm. in 33.6 cc. of 35% hydrochloric acid, diluted with water as noted below:

- a) 12½% zinc chloride and hydrochloric acid, 2 hours
- b) 25% zinc chloride and hydrochloric acid, 2 hours
- c) 50% zinc chloride and hydrochloric acid, 2 hours
- d) 75% zinc chloride and hydrochloric acid, 1 hour
- e) 100% zinc chloride and hydrochloric acid,  $\frac{1}{4}$ – $\frac{1}{2}$  hour

Exact length of time determined by degree of hydrolysis attained or desired.

4. Dehydrate in dioxan.

- a) 25% dioxan (three changes), 2 hours
- b) 50% dioxan, 1 hour
- c) 75% dioxan, 1 hour
- d) 100% dioxan and butyl alcohol, equal parts, three changes,  $\frac{1}{2}$  hour each. Add one drop of oil to each cubic centimeter of solution in the last change.

The writer has used cellusolve according to HANCE.<sup>2</sup> It shortens the operations and time occupied in 2, 3, and 4, but the results in staining have not been so satisfactory as with the longer schedule. It also aids in softening tissue and for some tissues may be suitable, but with filbert buds, etc., softening did not progress to the point where satisfactory sections could be consistently cut.

5. Put in vials and fill with shaved paraffin, place on oven over night and in oven in the morning. Pour off and add pure paraffin, changing three or four times and allowing  $\frac{1}{2}$  hour for each change. Avoid keeping in the oven longer than necessary for good infiltration of the paraffin, as this process hardens the hairs.

6. Treatment of paraffin blocks:

- a) Cut imbedded material into small blocks  $\frac{1}{8}$ – $\frac{1}{2}$  inch square and soak in water for several months. (For filberts the buds have been soaked for at least 6 months.)
- b) Remove from containers and place in open dishes containing a bacterial suspension, prepared by placing dead and decaying straw and grass in the dish with a solution of:

$\frac{1}{2}$  gm. ammonium nitrate  
1000 cc. water

Fill small dishes with this stock solution and place more straw and paraffin blocks therein. Keep in a warm room out of the sunlight for 2 or more weeks, adding more stock solution as evaporation progresses.

It has been impossible to cut suitable sections simply by soaking in pure water for 6 months, nor has it been possible to obtain satisfactory sections with the bacterial suspension for a few weeks alone. The combination of the two treatments, however, has

<sup>2</sup> New histological methods. Gradwohl Laboratory Digest. Vol. II, no. 9. 1937.

given excellent results. Whole buds can be sectioned at 10  $\mu$ . Of the pistils and attached tissues, sections 5-7  $\mu$  thick are readily cut without tearing.

The paraffin blocks are chilled in an icebath and cooled from time to time with blocks of ice as cutting progresses. The microtome knife must be sharp, and it is prepared for use according to directions given by EVENDEN and SCHUSTER.<sup>3</sup>

The following stains have been used successfully on filbert buds sectioned by the preceding schedule. Apparently the treatment does not greatly affect staining qualities of filbert tissues.

- a) Leaf buds.—Haematoxylin with one drop of tergitol no. 7 added to a jar of stain.
- b) Pistillate buds through pollinating season.—Orseilline B.B. and light green combination.
- c) Pistillate buds from beginning of embryonic tissue.—Orseilline B.B., fast green F.C.F., methyl violet 2B, and orange G, according to JOHANSEN,<sup>4</sup> except that orseilline B.B. is substituted for safranin O.

<sup>3</sup> A new sharpening back and procedure of sharpening for microtome knives. *Stain Technol.* 14:69-71. 1939.

<sup>4</sup> A quadruple stain combination for plant tissues. *Stain Technol.* 14:125-128. 1939.

## CURRENT LITERATURE

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*Modern Fruit Production.* By J. H. GOURLEY and F. S. HOWLETT. New York: Macmillan Co., 1941. Pp. vi+579.

This volume illustrates the great strides made in the last 30 years toward the merging of the abstract and the more closely applied aspects of plant science. The fields of ecology, physiology, and morphology are well represented in the chapters dealing with factors affecting flower formation, general cultural practices, water relations, fruit setting and thinning, nutrient deficiencies, the origin and improvement of fruits, etc.

The book is much more than a compilation. Many practices are analyzed in detail in the light of existent botanical knowledge, whereas others are less clearly defined. In this there seems to be no alternative possibility, for botanical investigation must be more penetrating far before anything approaching detailed analyses of field practices can be made. There might well be disagreement with some of the interpretations of the authors on the body of evidence considered; but it is their views which give distinctive character to the book.

The illustrations are relatively scarce, but in the main are informative and to the point. The reader will note the omission of much of the older standard literature, not a particularly serious loss, since—with some additions and modifications—many of the more recent contributions largely repeat and embody the same material. The entire book is pleasingly readable and thoroughly usable to the botanist, the horticulturist, and anyone seriously interested in fruit crops. It is a worthy addition to the slowly growing list of texts attempting a rationalization of agricultural practice in terms of progressive research.—E. J. KRAUS.

*Photosynthesis.* By E. C. C. BALY. New York: D. Van Nostrand Co., 1941. Pp. vi+248. Figs. 24. \$4.75.

In this third monograph to bear this title (two by other authors having appeared in 1926), BALY presents in full the various contributions he has made to the problems of photochemical synthesis of carbohydrates and nitrogenous compounds in the last 20 years. The work is somewhat narrow in its scope, since no serious attempt has been made to incorporate into the discussion the extensive research done in this country. The work of EMERSON and SPOEHR is included, but no other important American contributions are cited.

The early chapters, introductory, deal with the difficulties of interpreting photosynthesis from the standpoint of photodynamics and quantum relationships; they also include a retrospective examination of the early investigations on the action of ultraviolet radiations on solutions of carbon dioxide, on the polymerization of formaldehyde into glucose, and on the photosynthesis of carbohydrates by the action of light on hydrated carbon dioxide adsorbed on a surface. Chapter V discusses the final achievement of photosynthesis of carbohydrates, and deals with the more recent investigations. A chapter follows on the assimilation of nitrogen by the living plant. The last two chapters attempt to picture the mechanism of photosynthesis and the kinetics of the process. The author index lists slightly over 100 names, and a subject index contains about three pages.

Well written, easy to read and understand, this volume's main value is to bring together and summarize the author's own contributions and viewpoints. Some of these have aroused controversial discussion as to the adequacy of the experimental techniques and as to the validity of the conclusions, which in some cases have seemed highly hypothetical. The work should be welcomed and enjoyed by those who, keeping this in mind, can read it with well-balanced critical perspective. It should stimulate a valuable rethinking of the problems and a better experimental approach to the final solution and acceptance of a mechanism in full harmony with the kinetic requirements.—C. A. SHULL.

*Flowers and Flowering Plants.* 2d ed. By RAYMOND J. POOL. New York: McGraw-Hill Book Co. Inc., 1941. Pp. xxiii+428. Figs. 211. Folded chart. \$3.50.

The new material included in this edition was largely selected from suggestions made by teachers of plant taxonomy. A few minor rearrangements of the subject matter give the book greater coherence. A new chapter on the vegetative organs of plants, with particular emphasis on the terminology of leaf margins, tips, bases, etc., as well as the appended glossary, adds to the completeness of the text. Revision of the map indicating the useful ranges of plant manuals in the United States brings the reference matter up to date.

A noteworthy change is made in the folded chart facing page 159. The relative phylogenetic positions of the orders of flowering plants are shifted only slightly when compared with the chart of the first edition, but the families are omitted, except for some of the apetalous and anemophilous groups. A habit picture, a cross-sectional floral diagram, and a composite floral formula are given for each order named on the chart. Most teachers of systematic botany will applaud this simplification. Even though no adequate explanation of the method of applying scientific names to plants is given, this work is the most useful text in its field.—P. D. VOTH.

*Biological Symposia.* Vol. II. Edited by JAKES CATTELL. Lancaster, Pennsylvania: Jaques Cattell Press, 1941. Pp. 270.

This second volume consists of some 21 papers which were presented at the Milwaukee meeting of the American Association for the Advancement of Science, June 20, 1939; the Columbus meeting, December 28, 1939; and the meeting of the Western Society of Naturalists at Monterey, December 20, 1939.

The subject matter is presented under four sections: (1) speciation; (2) defense mechanisms in plants and animals; (3) biological basis of social problems; and (4) regeneration. The bringing together in one volume of such a group of scholarly papers, thus making them available to all interested, is a valuable service to science. The editor merits the commendation of all biologists.—J. M. BEAL.

*Hunger Signs in Crops. A Symposium.* Washington, D.C.: Judd and Detweiler (published by American Society of Agronomy and National Fertilizer Association), 1941. Pp. xiii+327. Illustrated. \$2.50.

A symposium on nutritional deficiency symptoms, presented in nine chapters, with fourteen individual contributors. Nearly 80 colored plates are used in illustrating the symptoms, and almost 100 uncolored figures. The first chapter is introductory, discussing why plants starve. The succeeding chapters take up specific crops, or closely related groups. The stories of starvation and response are told in simple, direct language. Literature references are not used. As the authors in every case are experts with the crops considered, the book is an authoritative

and accurate summary of nutrient deficiencies in our main agricultural crops. This work is an example of cooperation between science and industry, and because it is, it costs far less than if it had been produced wholly as a scientific venture. This book gives adequate information on a subject of widely growing popular interest, since correct plant nutrition is the basis of safe nutrition for animals and man. It is an outstanding contribution to popular understanding of an important field of plant physiology.—C. A. SHULL.

*Bibliography of References to the Literature on the Minor Elements and Their Relation to Plant and Animal Nutrition. Second Supplement to Third Edition.* New York: Chilean Nitrate Educational Bureau, Inc., 120 Broadway, 1941.

The second supplement to this important bibliography contains 67 pages; almost 500 references with brief abstracts; and the threefold index, by elements, botanical species, and authors. Almost 50 of the elements are listed, and approximately 150 species of plants—by their common names usually. The entire work is the most useful bibliography in the field of plant nutrition and constitutes an outstanding contribution to the research facilities of the plant physiologist. The Chilean Nitrate Educational Bureau deserves high commendation for continuing this vital service to plant science.—C. A. SHULL.

## FIFTIETH ANNIVERSARY CELEBRATION

\* \* \*

*In connection with the Fiftieth Anniversary Celebration of The University of Chicago, a Symposium on Growth and Differentiation in Plants will be held by the botanists, Monday morning, September 22, 1941.*

*Papers for the session will be contributed by*

DR. C. E. ALLEN, University of Wisconsin

DR. J. M. BEAL, University of Chicago

DR. E. W. SINNOTT, Yale University

DR. J. W. MITCHELL, U.S. Department of Agriculture

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